Drug Discovery in Infectious Diseases
Edited by Paul M. Selzer

Antiparasitic and Antibacterial Drug Discovery

From Molecular Targets to Drug Candidates

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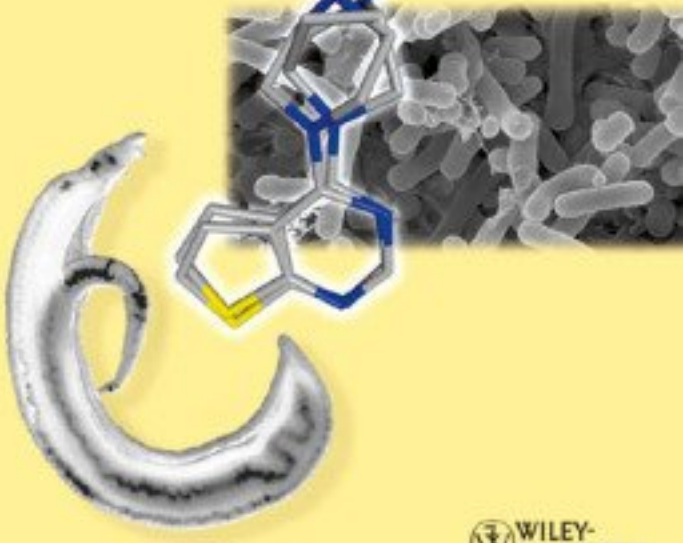

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Antiparasitic and Antibacterial Drug Discovery

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Antiparasitic and Antibacterial Drug Discovery

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Cover
Light microscopic image of the helminth *Schistosoma mansoni*–with a male hosting a female in the *canalis gynaecophorus*: courtesy of Dr. Conor R. Caffrey, University of California San Francisco, USA. Scanning electron microscopic image of the gram-negative bacteria *Mannheimia heamolytica*: courtesy of Prof. Dr. Lothar H. Wieler, Freie Universität Berlin, Dr. Heike Kaspar, and Dr. Christoph Schaudinn, Robert Koch Institut Berlin, Germany. The chemical structure is taken from chapter 19 authored by Thorsten Meyer *et al.*, figure 19.9.

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Foreword

It is ironic that three decades ago infectious diseases were viewed as a problem of the past. Malaria and tuberculosis were going to be eradicated, effective vaccines were available for major childhood infections, and an armamentarium of antibiotics was available for common community and hospital-acquired infections. Young physicians were advised not to enter infectious disease specialties because they were becoming irrelevant. The AIDS epidemic was the first wakeup call that infectious diseases would again become a major global health problem. Drug-resistant malaria and tuberculosis are now almost ubiquitous and new and emerging infectious diseases are almost a weekly staple of the popular press. Indeed the need for new drugs for infectious diseases has never been greater. Global industry and global travel means that formerly exotic diseases can rapidly establish themselves at any port of entry. Effective vaccines against the most prevalent infectious diseases like AIDS and malaria have proven difficult to develop. Multidrug-resistant organisms are an issue in any clinical setting. This publication provides a window on new approaches to drug discovery and development targeting infectious diseases. Fortunately, technology and training in new methodologies of drug discovery have expanded rapidly in the past 10 years. The challenge is how to effectively apply this technology to the thorny problems of global infections and to maintain a drug development pipeline for infectious diseases in light of the immense cost now associated with bringing new drugs to market.

San Francisco, USA
November 2008

James H. McKerrow

Contents

Foreword *V*
Preface *XI*
List of Contributors *XIII*

Part One Drug Discovery Approaches *1*

1 Target Identification and Mechanism-Based Screening for Anthelmintics: Application of Veterinary Antiparasitic Research Programs to Search for New Antiparasitic Drugs for Human Indications *3*
Timothy G. Geary, Debra J. Woods, Tracey Williams, and Solomon Nwaka*

2 Anthelmintic Resistance as a Guide to the Discovery of New Drugs? *17*
Georg v. Samson-Himmelstjerna, Roger K. Prichard, and Adrian J. Wolstenholme*

3 Drug Discovery for Neglected Diseases: View of A Public–Private Partnership *33*
Rob Don and Eric Chatelain*

4 Bioinformatics and Chemoinformatics: Key Technologies in the Drug Discovery Process *45*
*Andreas Krasky, Andreas Rohwer, Richard J. Marhöfer, and Paul M. Selzer**

5 Target Identification and Validation in Antiparasitic Drug Discovery *59*
*Christian Wolf and Nikolas Gunkel**

6 Selective Drug Targets in Parasites *75*
Peter Köhler and Richard J. Marhöfer*

7 **Lessons Learned from Target-Based Lead Discovery** *99*
*Michael Gassel, Jörg Cramer, Christopher Kern, Sandra Noack, and Wolfgang Streber**

8 **Approaches Towards Antiparasitic Drug Candidates for Veterinary Use** *117*
Christophe Chassaing and Harald Sekljic*

9 **Learning to Relate Structural Space to Property Space** *135*
Michael Berger, Jörg Cramer, Michael Hinz, Christina Mertens, Christian Miculka, Trevor Newton, Jörg Schröder, and Harald Sekljic*

10 **Recruiting the Host Defense Mechanisms: Roles for Vaccines and Chemotherapeutics** *159*
*Theo P.M. Schetters**

Part Two Protozoan Parasites *175*

11 **Proteases of Parasitic Protozoa – Current Status and Validation** *177*
Mohammed Sajid, Michael J. Blackman, Patricia Doyle, Chen He, Kirkwood M. Land, Cheryl Lobo, Zachary Mackey, Momar Ndao, Sharon L. Reed, Brian Shiels, Ryan Swenerton, and William Weir*

12 **In Search of Trypanocidal Drugs** *211*
*Leopold Flohé**

13 **Trypanosomatid Protein Kinases As Potential Drug Targets** *227*
*Martin Wiese, Andrew Morris, and Karen M. Grant**

14 **Targeting the Malaria Kinome: Discovering Kinase Inhibitors as Novel Antimalarial Agents** *249*
*Dayadevi Jirage, Susan M. Keenan, and Norman C. Waters**

15 **Malaria and Antimalarials – a Focused View** *277*
*Frank Seeber**

Part Three Multicellular Parasites *299*

16 **Chemotherapeutic Development Strategies for Schistosomiasis** *301*
Conor R. Caffrey, David L. Williams, Matthew H. Todd, David L. Nelson, Jennifer Keiser, and Jürg Utzinger*

17 **Searching New Antiparasitics in Virtual Space** *323*
*Frank Oellien, Kristin Engels, Jörg Cramer, Richard J. Marhöfer, Christopher Kern, and Paul M. Selzer**

18 **Cyclooctadepsipeptides – an Anthelmintically Active Class of Compounds Exhibiting a Novel Mode of Action** *339*
Achim Harder, Kathryn Bull, Marcus Guest, Lindy Holden-Dye, and Robert Walker*

19 **Chemical Optimization of Anthelmintic Compounds – A Case Study** *357*
Thorsten Meyer, Jörg Schröder, Manfred Uphoff, Sandra Noack, Anja R. Heckeroth, Michael Gassel, Petra Rohrwild, and Thomas Ilg*

Part Four Bacteria *373*

20 **Pathogenomics: Identification of Novel Drug Targets and Vaccine Candidates in Bacteria** *375*
Knut Ohlsen, Martin Eckart, Ulrich Dobrindt, Heike Bruhn, and Jörg Hacker*

21 **Tuberculosis Drug Discovery: Issues, Gaps and the Way Forward** *415*
Véronique Dartois, Franz Joel Leong, and Thomas Dick*

22 **Decreasing the Number of Gaps in the Draft Assembly of the *Mannheimia haemolytica* M7/2 Genome Sequence** *441*
*Hon Q. Tran, Mathias Beig, Volker Spehr, Andreas Rohwer, Gottfried Unden, and Paul M. Selzer**

23 **Total Synthesis and Configurational Assignment of Pasteurestin A and B, a Natural Product with Antimicrobial Activity on Pasteurellaceae** *453*
*Marion Kögl, Lothar Brecker, Ralf Warrass, and Johann Mulzer**

Index *473*

Preface

In the age of antibiotics, vaccines, and drugs, we might be lulled into a sense of complacency regarding infectious diseases and that there is "a cure for everything". This sense of security is maintained at our peril, however. One has only to consider the growing devastation caused by such big-name diseases as influenza, HIV-AIDS, tuberculosis, and malaria to see that the struggle to treat and control infectious diseases is truly titanic and indeed becoming more perilous with the ever-evolving development and spread of drug resistance compounded by the greater freedom and speed of movement of goods, animals, and people. Aside from the recently perceived security threat to the health and business structures of the developed world caused by these and a plethora of other infectious disease, billions living in developing countries must endure the daily struggle of diseases. In contrast to most human health-related pharmaceutical companies, academic institutions, veterinary science, and animal health companies remain very much focused on infectious diseases, including those caused by bacteria and parasites. As illustrated in Figure 1, the animal health sector remains profitable, and thankfully so, as history has shown that therapies produced in this sector often prove invaluable for treatment of similar infectious diseases of humans – the application of anthelmintics being a case in point.

The improved understanding of the resilience of disease-causing agents to therapies, their expanding disease menace in the era of "globalization," and the balance provided by the opportunities for cross-sector exchange of ideas and applications spurred the preparation of this book. Also, the book serves to highlight the importance and visibility of drug discovery efforts for infectious diseases of both animals and humans.

Though it is not possible to address every aspect, disease, or approach within a single volume, this book sets forth a series of case studies and review articles that focus on bacterial and parasitic diseases in order to showcase how scientists in the different disciplines strive to move drug discovery forward. The contributing authors are experts drawn from drug discovery units of the pharmaceutical industry, academia, and nonprofit organizations in an effort to offer a global and balanced insight into the issues and problems at stake and their possible solutions.

Writing this has been a rewarding task for everybody involved. My heartfelt thanks go to the contributing authors for their excellent work performed within a short timeframe. In addition, I am grateful to Intervet/Schering-Plough Animal Health and its Drug Discovery Unit for their unreserved support, inspiration, and motivation

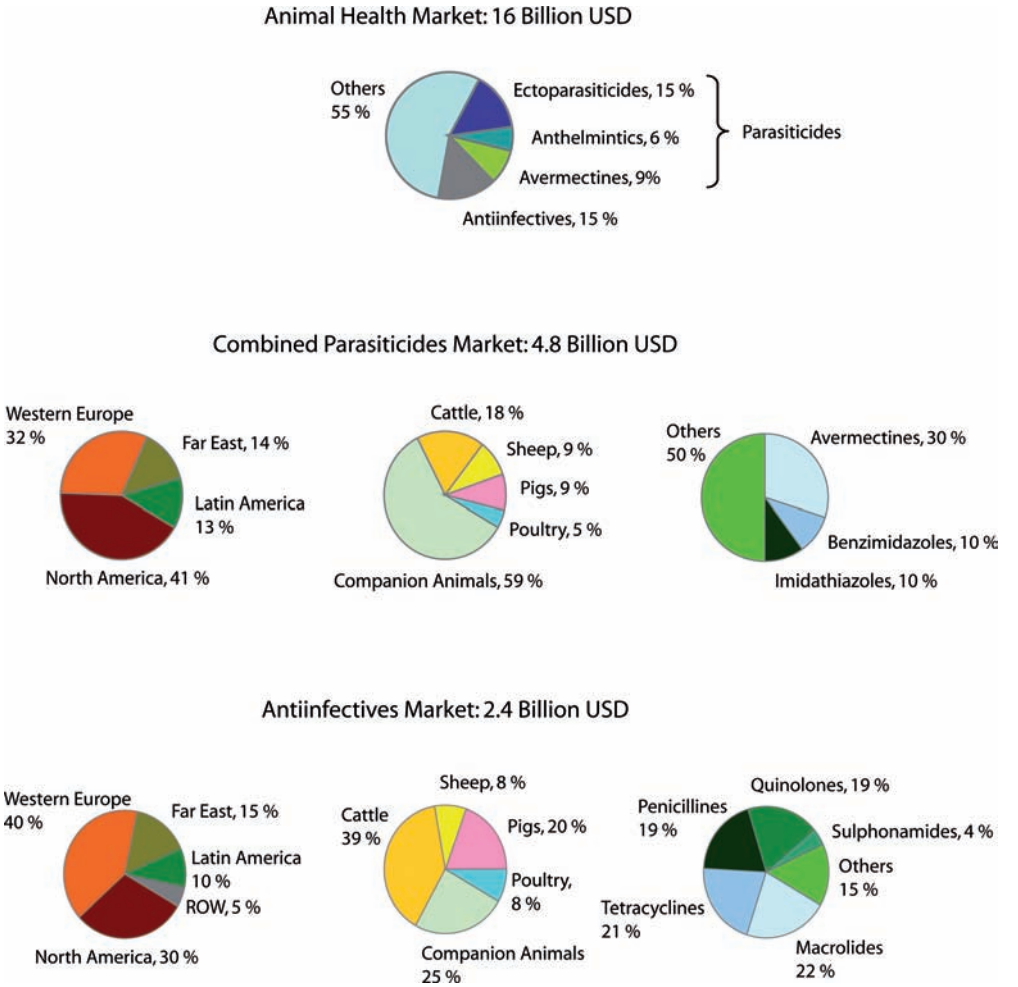

Figure 1 The world animal health market based on data from 2006. The first row depicts the proportion of antiparasitics and antiinfectives in the whole animal health market. Rows two and three represent the antiparasitics and antiinfectives market, respectively. From left to right the individual proportions are broken down according to regional sales (ROW = rest of world), sales per animal species, and sales per chemical class. The area of the individual pie charts is not size-adjusted. Original data were derived by Wood Mackenzie and kindly provided by Linda Franken-Horspool, International Marketing, Intervet/Schering-Plough Animal Health.

during the preparation of this book. I also thank the members of Intervet's Bio-ChemInformatics Unit for their excellent technical backing and team spirit. Finally, I am very grateful to Ms Simone Maus-Gilbert for her outstanding editorial assistance.

Schwabenheim, Germany *Paul M. Selzer*
November 2008

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Part One
Drug Discovery Approaches

1
Target Identification and Mechanism-Based Screening for Anthelmintics: Application of Veterinary Antiparasitic Research Programs to Search for New Antiparasitic Drugs for Human Indications

Timothy G. Geary, Debra J. Woods, Tracey Williams, and Solomon Nwaka*

Abstract

Anthelmintic discovery in the veterinary pharmaceutical industry has succeeded only through screening synthetic compounds and fermentation products against whole parasites in culture or in host animals. Following trends in the parent, and much larger, human pharmaceutical industry, many programs have been developed in the past 20 years to exploit mechanism-based screening strategies for the identification of new leads in this therapeutic area. This strategy relies on the robust identification of parasite proteins as targets for chemotherapeutic intervention and their subsequent validation. Expanding access to sequenced genomes of parasitic nematodes will facilitate identification of genes that encode putative drug targets. Of particular relevance will be those that are shared among nematodes of veterinary and human importance. These targets offer the best chance for finding new molecules with potential utility in both arenas and provide an opportunity for collaboration and synergy between the two sectors. Validation of these gene products as drug targets will require advances in functional genomics methods for parasites. Expanded capacities for parasite-based physiological and biochemical experiments are also likely to be needed. While mechanism-based approaches remain an attractive alternative to organism-based strategies for broad-spectrum anthelmintic discovery, proof-of-concept for the platform is still needed.

Introduction

Screening for antiparasitic drugs as a scientific exercise can be traced to the early work of Ehrlich, who screened a collection of synthetic dyes for trypanocidal activity in mice with the aim of allowing the importation of European horses and

cattle into the African colonies of Germany prior to 1900 (see Refs. [1, 2]). This was perhaps the first example of a screen of a collection of chemicals for any therapeutic indication; Ehrlich's efforts at drug discovery seem to have begun with a veterinary parasite as target, but led to the introduction of the first anti-infective drugs for use in humans. Thus, the process by which drugs introduced into veterinary practice for parasite control were adopted for use in humans has a long history.

The motivation to discover "modern" antiparasitic drugs for the animal health industry can be traced to the introduction of sulfaquinoxaline for the prevention of mortality and morbidity due to poultry coccidiosis in the late 1940s, phenothiazine (1930s) and piperazine (1950s) as veterinary anthelmintics, and the chlorinated hydrocarbons and organophosphates as ectoparasiticides in the 1940s and 1950s. Diethylcarbamazine was discovered as an agent for use in human filariasis within the same time-frame, being developed for veterinary practice for heartworm prevention some time later. It is important to note that all these drugs were first used in humans – not necessarily for parasites – prior to being adopted for veterinary use. Their utility for controlling parasites in animals paved the way for the institution of systematic screening of chemical collections for new synthetic antiparasitic drugs for veterinary application. Their use in clinical settings proved that chemotherapeutic control of parasites which plagued livestock and poultry was economically rewarding for the manufacturer, the veterinarian, and the farmer.

The general flow of antiparasitic drugs from human to veterinary application (Table 1.1) reversed over time. For anthelmintics, the reversal began with the discovery of thiabendazole for veterinary medicine in the 1960s (Table 1.2), which was later introduced for treatment of various gastrointestinal (GI) nematodes in humans. This pattern was repeated for the nicotinic cholinergic agonists (pyrantel, levamisole), the second generation benzimidazoles, particularly albendazole and mebendazole, and ivermectin (and, potentially, related macrocyclic lactones). In contrast, antiprotozoal drugs have not moved as easily between the sectors (Table 1.2) or continue to flow in the opposite direction, a situation that primarily reflects the

Table 1.1 Antiparasitic drugs introduced to veterinary practice from human medical interest.

Drug	Human use	Veterinary use
Diethylcarbamazine	Filariasis	Heartworm prophylaxis
Arsenicals	Trypanosomiasis, onchocerciasis	Heartworm therapy
Piperazine	Gout (discontinued)	GI nematodes
Phenothiazine	Malaria/mosquito control	GI nematodes
Sulfa antibiotics	Bacteria	Poultry coccidiosis
Metronidazole	Anaerobic microbes	Giardiasis
Buparvoquone	Malaria	Theileriosis
Halofuginone	Malaria	Poultry coccidiosis

Table 1.2 Antiparasitic drugs introduced into human use from veterinary interest.

Drug	Veterinary indication	Human indication
Benzimidazoles	Anthelmintic/antiprotozoal	Nematodes, protozoa
Pyrantel/levamisole	GI Nematodes, Lungworms	Nematodes
Praziquantel	Cestodes	Schistosomiasis
Ivermectin	Heartworm prophylaxis	Filariasis
Nitazoxanide	Sarcocystis in horses	Protozoa, nematodes
Moxidectin	Nematodes, ectoparasites	Onchocerciasis (in development)
Emodepside	Nematodes	Onchocerciasis (investigation)

differences in the major species of protozoal pathogens of animals compared to humans (see below).

Potential for Veterinary → Human Transfer of new Antiparasitic Drugs

Like most of the pharmaceutical industry, animal health companies underwent a considerable reduction in abundance over the past 20 years from mergers and acquisitions [3, 4]. This led to a net reduction in investment in antiparasitic drug discovery, with a consequent focus of efforts on the most profitable sectors of the animal health market [5]. As a result, priorities for veterinary parasite control now diverge more extensively from those of human medicine. A summary of current emphasis on types of parasites targeted for drug discovery for human versus veterinary applications is shown in Box 1.1. It is worth noting in this context that there may be a

Box 1.1: Areas of synergism/overlap based on current trends in discovery investment

Apicomplexan protozoa:	human ↑, veterinary ↓
Kinetoplastids:	human ↑, veterinary ↓
Giardia/ameba/*Cryptosporidium*:	human ↓, veterinary ↓
Trematodes:	human ↔, veterinary ↔
Filarial nematodes:	human ↑, veterinary ↔
GI nematodes:	human ↓, veterinary ↑

This box illustrates the potential for flow of compounds in each direction as discovery efforts continue.
↑: relatively high interest and activity in discovering new drugs.
↔: modest interest/activity.
↓: minimal or declining interest/activity.

resumption of drug transfer for parasites from the human to the veterinary side in the future. This situation may benefit both areas, as described below.

Protozoan Parasites

A renaissance has occurred in the attention of public and private funders to the discovery of new drugs for protozoal parasites that infect humans. The primary targets for chemotherapy include the Apicomplexan malaria parasites (*Plasmodium* spp.), kinetoplastids such as *Leishmania* spp., *Trypanosoma brucei* and *T. cruzi*, *Entamoeba histolytica*, *Giardia lamblia*, *Toxoplasma gondii*, *Trichomonas vaginalis*, and *Cryptosporidium parvum*. Based on prevalence and pathogenicity, these drug discovery efforts are considerably weighted to malaria and the kinetoplastids [6–12]. In contrast, the primary protozoal target for veterinary medicine is a distinct group of Apicomplexans, the *Eimeria* spp. of poultry, with additional interest in phylogenetically related parasites (*Neospora caninum*, *Sarcocystis neurona*) and in *Giardia* spp. and *Cryptosporidium* spp. [3]. However, dedicated antiprotozoal discovery programs are no longer common in the animal health pharmaceutical industry (vaccine discovery is more prevalent at this time), and so future drugs for these infections will likely flow from human to veterinary use. Current work in this area on the human side is heavily focused on mechanism-based, as opposed to whole-organism, high-throughout screening. The extent of target overlap is likely to be reasonably good across the human/veterinary species divide, though target choice in the human-focused projects does not routinely include an assessment of relevance for parasite species of strictly veterinary importance. Inclusion of this factor as a criterion for prioritization could provide a for-profit component that would appeal to potential animal health partners, with benefits similar to those anticipated in the anthelmintic arena (see below).

Ectoparasites

Indications for the use of ectoparasiticides in human medicine are far fewer than for veterinary clientele, which in turn is a much smaller market than agricultural applications. The flow of these compounds has typically been from agriculture to animal health to human applications, with the exception of DDT, which was first developed for use in humans. The use of ivermectin for the treatment of head lice and scabies is an example of an ectoparasiticide developed for animals being adopted for humans. However, the current economic driving force for this arena is so small that discovery programs in animal health sectors typically do not include a component that addresses possible human uses. From the human medical perspective, the temporally limited (as opposed to chronic) use of these products and the relatively low number of infestations in the West make the cost–benefit analysis in terms of registration unrewarding. This situation may change if head lice and scabies develop more extreme resistance to available ectoparasiticides, including ivermectin.

Trematodes

These considerations suggest that the primary influence of animal health drug discovery research on human medicine will continue to be in the anthelmintic arena. More specifically, this will be largely restricted to drugs that primarily affect nematodes. The only flatworm of economic significance in veterinary medicine is the liver fluke, *Fasciola hepatica*. This parasite is important in some areas, but is not enough of a production problem in livestock to warrant dedicated screening in most animal health companies, even though resistance is emerging to the best available drug, triclabendazole (which is not even registered in the USA). Although *F. hepatica* is a significant human pathogen in some regions, it has not proven to be sufficiently prevalent to elicit a dedicated discovery effort for it. Instead, work on flatworms in the human sector focuses on *Schistosoma* spp., currently controlled by a single drug (praziquantel). In the absence of rigorously documented cases of praziquantel resistant schistosomes, investment in new antischistosomal drug discovery has been somewhat limited compared to the efforts mounted against protozoa. This situation may be changing in light of the Helminth Drug Initiative recently developed by WHO/TDR [13], which aims to reinforce and advance screening for new antischistosomal drugs. As for protozoan parasites, this effort may discover compounds that can be adopted for use against liver flukes in veterinary medicine.

Nematodes

Further analysis of the impact of veterinary antiparasitic drug discovery programs will be restricted to nematocides. Historically, the discovery of nematocides for use in animals or humans was based on low-throughput systems that employed infected animals as the primary screen. These assays were labor-intensive, slow to attain the final read-out and used relatively large amounts of experimental compounds. Even so, it remains true that at least the prototype of every available anthelmintic class, including emodepside, the paraherquamides, and the newest class, the AADs, was discovered by screening in infected animals or worms in culture. Nonetheless, there has been a marked shift of strategy in the animal health industry to emphasize discovery programs based on targets, or high-throughput, mechanism-based screening [14].

The initial change from screening in infected animals to tests run on organisms in culture was motivated primarily by the need for animal health operations to fit into the evolving discovery paradigms adopted by their parent companies. This meant a marked reduction in the amount of chemicals used in a screen (to adapt to new parameters for compound synthesis in medicinal chemistry programs) as well as a reduction in animal use and in labor costs associated with screening. In addition, there were concerns that *in vivo* screens might fail to detect truly interesting actives that are false negatives due to insufficient potency or pharmaceutical inadequacy.

Unfortunately, screening against parasites or the free-living species *Caenorhabditis elegans* in culture, while vastly increasing throughput and radically diminishing the amount of compound needed for primary screening, was not very successful in revealing new anthelmintic templates. Indeed, these procedures led to a very high rate of false positives, as many compounds were noted to kill nematodes in culture, but very few (almost none) were subsequently found to be active in infected animal models. As resources were typically insufficient to permit experiments designed to determine why *in vitro* actives routinely failed *in vivo*, improvements in the screening stream designed to reduce the incidence of false positives were not possible. A new approach was clearly needed, and it was incompatible with standard industry practices to return to the era of screening in infected animals. In keeping with drug discovery for human medicine, mechanism-based approaches came into vogue [14].

The drive to move from organism to target-based screens was based on several factors. One factor was the motivation to capitalize on biology-based intellectual property (IP); screens using infected animals or organisms in culture barred few competitors from an area and can only exploit chemistry-based IP. Mechanism-based screens can restrict the discovery activities of competitors by taking advantage of investments made in understanding the physiology and molecular pathology of diseases and infectious pathogens that dominate Western human medicine. In addition, advances in chemical technology, such as combinatorial chemistry, made it possible to synthesize thousands of molecules at a time in small amounts; organism-based screens were ill-equipped to test either the number or the small amount of available compounds. Advances in computational chemistry and structure-based drug design meant that the traditional whole-organism blind-screen approaches became seen as intellectually unchallenging and out of step with the times. Finally, whole-organism approaches are labor-intensive compared to mechanism-based strategies; the incorporation of mechanism-based screening allowed a relatively small team of screeners to evaluate hundreds of thousands of compounds against multiple targets in a matter of weeks. The combination of vastly increased throughput with lowered labor costs made this an irresistible strategy, validated by expert scientific opinion. To date, however, it is undeniably true that the adoption of this overall strategy has not led to an increase in the number of new chemical entities registered for use in humans. For our topic, it is crucial to stress again that at least the prototype of all commercially available anthelmintics was discovered in whole-organism assays, despite at least two decades using the more modern approaches to discovery. What does this bode for the switch to more modern screening platforms? We can use the neuropeptide area as an example (see page 12).

Discovery Synergies

Discovery programs in animal health companies can contribute to the discovery and development of drugs for use in humans in several ways. The most obvious is the

direct transfer of veterinary-approved drugs to human medical use, as was the case for ivermectin and albendazole, among others. It may happen again with compounds such as moxidectin or emodepside. In these cases, human clinical trials are still required for registration, but much of the basic registration package will already be available. It should be noted that this transfer does not directly benefit the animal health partner.

Failing that happy occurrence, another synergy can occur through the donation of selective compound collections to discovery efforts targeted at human parasites. Most animal health companies have been engaged in antiparasitic drug discovery for decades and have assembled a set of compounds with activity at some level of screening. The set may consist of thousands of compounds. These compounds were not commercialized for a variety of reasons, but they offer an enriched set of potential leads that can be tested in screens relevant for human parasites, either in mechanism-based assays or against whole organisms in culture. Such a transfer has occurred already as part of a TDR program to incorporate contributions from industrial sources into drug discovery processes [10, 11, 13]. A key component in the transfer is the negotiation of an agreement on IP for the compounds, which has been accomplished by TDR in several cases that can serve to facilitate additional agreements with other companies [10]. Activity of these "set aside" compounds in a human parasite-targeted screening stream may reawaken interest in the template in the animal health partner, providing a potential economic return on the collaboration.

This factor is particularly relevant in light of the recent history of anthelmintic discovery in the animal health industry. The extraordinary clinical utility of ivermectin and subsequent macrocyclic lactones set the bar for commercial introduction of new anthelmintics to the veterinary market at a very high level [3, 4]. This led to economic decisions in animal heath companies to shelve molecules that were potentially useful but clearly inferior to the macrocyclic lactones discovered during the course of screening. Increasing concerns about macrocyclic lactone resistance in veterinary medicine has led to renewed interest in these shelved molecules; as research into them is revived, new leads may emerge from once-discarded molecules or templates.

In the most basic example of potential synergy, animal health programs can contribute screening models and expertise to human parasite discovery efforts. These operations would screen the chemical collections of the industrial partner, providing unprecedented and otherwise unattainable access to chemical space for a human parasite target. In addition to screens employing whole organisms (parasitic or free-living), high-throughput, mechanism-based assays for targets of broad interest in nematodes (for other kinds of parasites as well) can be operated cooperatively. Expertise in design, performance, decision strategies and information management for such screens can be contributed by the animal health partner. Hits identified in the screen can be of interest to both partners or to either separately, depending on activity in subsequent screens. Importantly, agreements with animal health companies enable the onsite training of external scientists in this technology, which can broaden its expert use in non-industrial centers [10].

Post-Discovery Synergies

The conversion of hits from a high-throughput, mechanism-based screen to a legitimate lead compound with activity in animals against target parasite species has rarely (if ever) been attained. This is also a significant issue for drug discovery in other therapeutic areas [15, 16] and represents a major concern for the current discovery paradigm. Whereas it is quite easy to propose targets for mechanism-based screens, it is a very difficult thing to convert a target to a molecule with activity *in vivo*, let alone a drug. The multidisciplinary studies needed to generate a lead from a hit are commonly undertaken in industrial settings; the internal expertise in drug metabolism, pharmacokinetics, pharmacodynamics, formulation, and pharmaceutical chemistry is difficult (though not impossible) to assemble outside of a for-profit institution. The availability of this expertise and the associated experimental platforms and systems in animal health companies can be an enormous benefit to lead generation for human parasite indications if hits can be generated through screening. This is especially important for testing of compounds against parasites in animal models. The early phases of hit-to-lead generation overlap for a compound with activity against nematodes of both human and veterinary medical importance, representing a significant cost benefit for the human-oriented program.

Despite advances in high-density chemical synthesis for analog generation, medicinal chemistry remains the largest expense in drug discovery. In some respects, the role of screening and subsequent bioassays is simply to focus a company's medicinal chemistry resources on the most promising hits or lead series. Medicinal chemistry is an asset that must be applied in any lead-finding program. The most advanced research in this area is based in industry, and the cooperation of animal health companies in providing facilities and training for sponsored scientists to work in this milieu has great benefits [10]. Again, negotiating an agreement on ownership of and rights to compounds produced by the sponsored scientists are critical to ensure full access to the industrial expertise and facilities, but these have been successfully executed.

Additional contributions can be realized from mutually beneficial early development programs. This is unquestionably the case if the same compound is selected for use in humans and animals, but much can be gained from a shared effort even if different compounds are chosen for the two indications. Preliminary pharmaceutical chemical work (stability), advanced formulation, ADME, and toxicology will have at least some overlap. Importantly, research in process chemistry and manufacturing can be integrated, as both therapeutic areas demand the lowest cost of goods possible. Insofar as data from a veterinary-driven development program can be used to support development and registration of a compound for human use, there will be a significant cost savings in the human component; this may tip the balance sheet in favor of development by lowering the cost-recovery break-even point.

Although direct discovery of antiparasitic drugs intended primarily for use in humans is not a primary goal for major human pharmaceutical companies in the

West, work on neglected tropical diseases does occur in this sector [11]. Considerable expertise in running human clinical trials is available in these organizations and at least some have pursued or are pursuing development of existing drugs for use against human parasites (e.g., azithromycin for malaria [17]). The ability to potentially tap into these resources as part of an advanced development and registration collaboration involving the animal health component of a major pharmaceutical company is a substantial attraction to such arrangements.

As discussed above, compounds identified in screening operations for parasites of human interest (malaria or schistosomes in particular) may be adopted by animal health companies for development for the treatment of Apicomplexan parasites or trematodes of veterinary importance. Collaboration in the development stream illustrated for anthelmintics would provide similar benefits for leads flowing in the opposite direction and could favorably affect the costs of development that might otherwise limit or restrict the human-oriented pathway. This kind of interaction could be facilitated by the negotiation of agreements to funnel antimalarial or schistosomicidal actives to an animal health company for evaluation. Whether this kind of agreement is possible would depend to some extent on IP issues pertaining to the ownership of compounds screened for activity against human parasites. The potential of a revenue stream based on royalties from veterinary sales should not be discounted, even if the amounts are not likely to be large.

Cautions

Genomics-based approaches are a natural point of overlap between veterinary and human parasiticide discovery. Efforts to deduce useful anthelmintic drug targets by genome-based analysis [18–25] are of great potential value. However, there are several caveats to the adoption of this strategy in addition to the failure to demonstrate proof-of-concept to date for antiparasitic drug discovery (no marketed products discovered by this approach).

Perhaps the greatest concern is that little research on the biology of potential drug targets has been done in nematodes. Parasitic species are difficult to maintain in the laboratory, and functional studies on the physiology and biochemistry of these organisms have never been abundant and are decreasing in frequency. The plethora of targets identifiable in genomics approaches are difficult to prioritize by functional significance in the target organisms. This conundrum is particularly relevant for protein targets of uncertain function, based on annotation.

A second concern is that a fairly large proportion of nematode genes seem to be species-specific [26]. In the veterinary realm, this is not too critical, since breadth of spectrum is an essential feature for commercial success and thus species-specific targets are not pursued. However, targets unique to adult filariae may be worthy of

pursuit, since narrow-spectrum drugs for the control and potential eradication of lymphatic filariasis and/or onchocerciasis (river blindness) would be of high value – from a public health, if not economic, perspective. The lack of facile functional genomics approaches for parasitic nematodes means that validation of potential drug targets is only possible if they are present and serve the same role in *C. elegans*. Genes unique to filariae clearly fall outside of this category, as do genes that may encode proteins necessary for parasitism in general in this phylum [27]. At this time, there are no readily available techniques for validating such proteins as drug targets.

Another concern pertains to the difficulty of prioritizing candidate drug targets by biological validation. Such efforts rely primarily on methods to knock-down the function of target genes in *C. elegans*, primarily by RNA interference [19, 20, 25]. Such techniques are not reliably available in parasitic nematodes [28]; and their full exploitation will require labor-intensive work in animal models (which are not available for key species in human medicine). Another problem is that it is quite difficult to analyze gain-of-function changes in target proteins even in *C. elegans*. There are no convenient methods for genome-wide protocols to induce gain-of-function mutations. However, most currently marketed anthelmintics are agonists, the effects of which cannot be duplicated or predicted by RNAi. This is also true for newer anthelmintics; emodepside opens a K^+ channel [29] and the AADs are atypical nicotinic cholinergic agonists [30]. Neither target would have been prioritized for screening based on an RNAi filter. More basic research on nematode biology is needed to provide a robust platform for prioritizing drug targets.

A final concern is that targets are typically highly valued for screening if homologs are absent from mammals. This strategy is, on the surface, highly rational as it may help assure parasite–host specificity. However, commercially available anthelmintics target GABA receptors, nicotinic acetylcholine receptors, and β-tubulin, which are very highly conserved indeed; and macrocyclic lactones target glutamate-gated Cl^- channels but are also excellent agonists of their GABA-gated relatives. A discovery strategy that would have failed to discover most of the commercially available drugs in this class should be endorsed with great caution. It should also reinforce faith in the power of medicinal chemistry to safely exploit even very small differences in protein structure.

An Example

A project conducted at Upjohn/Pharmacia/Pfizer illustrates some of the issues that pertain to the adoption of mechanism-based screening in anthelmintic discovery. As noted, relatively little basic research has been done on parasite biology, especially in nematodes, with the goal of identifying drug targets. As a consequence, the animal health industry had to conduct this research itself. This is in marked contrast to the situation in industrial drug discovery for major human indications, for which

governments and other institutions fund a great deal of research, the results of which can be readily translated into mechanism-based, high-throughput screens by companies.

Beginning in the 1980s, research on nematode neurobiology revealed the presence of a family of neuropeptides related to the snail tetrapeptide FMRFamide that were very widely distributed in nematodes and other invertebrates, but were rare in vertebrates (see Ref. [31] for review). Based on their distribution and very potent neuromuscular physiology (primarily studied in *Ascaris suum*), the receptors for these FMRFamide-like peptides (FLPs) were chosen for high-throughput screening to identify non-peptide agonists or antagonists for evaluation as candidate anthelmintics [32]. A very-high-throughput screening campaign more than 15 years after inception of the program identified a relatively small number of validated hits [33], none of which have advanced to the market. Until an anthelmintic is commercialized, from this screen or from another mechanism-based approach, one must consider the strategy an unrewarded experiment to date.

The consequences of failure in a screening campaign can be profound, given the disparity of resources available in animal health companies compared to human-focused companies. The resources needed to develop and implement a mechanism-based screening program, from physiology to bioinformatics/genomics to screen construction, can represent a significant proportion of the typical R&D budget of an animal health company. This situation may discourage investment on HTS systems for veterinary targets and requires that such programs be integrated with less costly and potentially less risky whole-organism approaches. It also motivates collaboration between human and veterinary medical programs to spread the investment risk as broadly as possible. It is essential to recognize that, as long as parent pharmaceutical companies rely on mechanism-based screening approaches for drug discovery, animal health companies must participate in the process. The crucial task is how to ensure the best chance for success.

Conclusion

Bidirectional flow of antiparasitic compounds, leads, and hits between animal health companies and organizations devoted to discovery of drugs for human parasitoses offers many mutual benefits. For protozoa and trematode infections, the direction of this flow in the near term is likely to be from human to veterinary indications. The existence of multidisciplinary expertise in animal health companies can benefit the human development path if the same or similar compounds are chosen for advancement in the two fields. For nematocide discovery, synergies between the two areas are evident from target identification to screening to development toward registration. Fortunately, the benefits of collaboration with industrial partners have reinvigorated current efforts to discover new anthelmintics [10]. The results of this new enterprise are eagerly awaited.

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2
Anthelmintic Resistance as a Guide to the Discovery of New Drugs?

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Abstract

The issue of anthelmintic resistance has nowadays reached each drug class on the market longer than 10 years. Furthermore, it has developed to serious levels in some industries, for example, the sheep industry in the southern hemisphere. This has led to considerable research activities directed at the investigation of the mechanism of anthelmintic resistance, particularly in parasitic nematodes. An enormous amount of data has thus been generated on both specific and nonspecific phenotypical, functional, and molecular aspects of resistance. Three major anthelmintic drug classes (benzimidazoles, macrocyclic lactones, and nicotinergic agonists, i.e., imidazothiazoles, tetrahydropyramidines) have been in use for more than 25 years. The mechanism of action for each of these drug classes is now generally well understood, involving interference with parasite metabolism (microtubule assembly) and the nervous system (nicotinergic and glutamergic/GABAergic receptors). Based on this knowledge, studies on target specific mechanisms of drug resistance have elucidated that benzimidazole resistance can be correlated with changes in β-tubulin coding sequences. For the other drug classes this has not been unequivocally shown yet. In addition to the specific, target-directed resistance mechanisms, nonspecific mechanisms were also described, for example, based on the modification of drug efflux mechanism such as P-glycoprotein function, or based on drug metabolism such as cytochrome P450. This paper initially gives a brief update on anthelmintic resistance mechanisms, followed by a description of recent successes in order to develop possible future implications for anthelmintic resistance guided drug discovery.

Introduction

Anthelmintics currently are the cornerstones of the control of human and veterinary helminth infections and, due to the general lack of immunological alternatives, will

probably remain so for the foreseeable future. Their impressive efficacy (with the standard being more than 90% worm reduction), their overall excellent tolerability, the broad spectrum of affected species, and the low costs of the modern anthelmintics were the characteristics which led to the great success story of chemical worm control in livestock and companion animals during the past five decades. Current mass treatment campaigns against helminth infections led by the WHO to combat, for example, gastrointestinal worm infections in tropical countries are also based on the use of drugs employed for decades in the veterinary field [1, 2]. In the latter, however, serious and often dramatic levels of anthelmintic resistance are being recorded, mainly in ruminant and horse gastrointestinal nematodes but also in others such as liver fluke [3, 4]. The situation is now further complicated by more than two decades lack of new mode-of-action drugs for nematode control in livestock. It is therefore one of the key parasitological research issues to identify new anthelmintic drugs and develop the next product to at least maintain the current status of helminth control. Traditional strategies leading to the discovery of anthelmintic drugs were mainly based on the screening of candidates using *in vitro* and *in vivo* test systems, lately involving high throughput testing of large chemical libraries. This was meanwhile accompanied by numerous investigations aiming at the elucidation of the mode of action of drugs by using worms resistant against the respective drug class [5]. The most recent example has been the presentation of the aminoacetonitrile derivatives (AAD) as a novel drug class, apparently using a different mode of action than those described for the currently commercially available anthelmintics [6].

Mode of Action

Structural or Metabolic Drug Targets

Microtubules in eukaryotic cells play essential roles, such as intracellular trafficking, cellular absorption and secretion, anchoring membrane receptors at specific locations (e.g., at synapses in nerve cells), mitosis and meiosis, cellular architecture (e.g., the elongation of axons), and the migration of cells via cilia and cell pseudopods [7]. It is therefore, perhaps, not surprising that β-tubulin and microtubules, which are formed by polymerization of α/β-tubulin dimers, are the targets for a large number of pharmaceuticals [8]. Typically drugs that target tubulin or microtubules either cause instability in microtubules (e.g., colchicine, vinca alkaloids, benzimidazoles, etc.) or cause microtubules to become excessively stable (e.g., taxol). Microtubules are dynamic polymers with a growing end where additional α/β-tubulin dimers can be added and a loss end where α/β-tubulin dimers disassociate from the polymer. The process of adding tubulin dimers at one end of the microtubule and losing tubulin dimers from the other is termed treadmilling. It is believed that benzimidazole anthelmintics (which include thiabendazole, mebendazole, albendazole, oxibendazole, fenbendazole, oxfendazole, and the pro-benzimidazoles

such as febantal and netobimin) bind to either the α/β-tubulin dimer or to the growing end of microtubules, "capping" the microtubule so that additional dimers cannot be added at the growing end of the polymer. At the same time, at the loss end of the microtubule, α/β-tubulin dimers continue to be lost. In this way, if the microtubule is capped, the structure disappears within the cell, disrupting the many functions performed by microtubules, and leading to cell death. The exact dimensions of the benzimidazole binding site have not been unequivocally determined [9–11]. However, allelic changes in nematode β-tubulin at either codon 200 (phenylalanine to tyrosine), codon 167 (also phenylalanine to tyrosine) or codon 198 (glutamate to alanine) result in benzimidazole resistance [4, 10, 12–16] and a loss of high affinity benzimidazole binding [10, 17].

Neurotransmitter Receptors

The nervous system provides a rich source of targets for the control of agricultural pests and metazoan pathogens; and parasitic nematodes are no exception. In particular, the ligand-gated ion channels that mediate fast neurotransmission have been very successfully exploited by the agrochemical and animal health industries: both the largest selling insecticide (imidacloprid) and anthelmintic (ivermectin) target members of this superfamily. The reasons for this are several: nervous system function is essential for viability and interfering with synaptic transmission can have rapid effects on the target organisms. Though the superfamily is conserved throughout the animal kingdom [18], its pharmacology is enormously varied because many of the receptors are heteropentamers and possess a plethora of modulating and allosteric binding sites. As a result, it has been possible to develop compounds that are specific for the nematode receptors, with little effect on the host. The ligand-gated ion channels can be divided into two categories, those that are permeable to cations, allowing sodium and sometimes calcium to enter and depolarize the cell, and those permeable to anions, allowing chloride to enter and, usually, hyperpolarize the cell.

In addition to the drug classes acting directly on ion channels, there are the cyclooctadepsipeptides, whose mode of action was suggested to involve binding to G protein-coupled receptors and activation of calcium-activated voltage-gated potassium channels, leading to atonic paralysis [19, 20]. This drug class has provided the most recent new commercially available anthelmintic drug, emodepside, currently only released for the treatment of worm infections in cats. An ion channel-mediated mode of action is also proposed for the cestocidal drug praziquantel: sensitivity to which is conferred by a helminth-specific β-subunit of a voltage-gated calcium channel, which leads to calcium influx, causing muscle contraction and paralysis [21]. However, praziquantel resistance is currently still an occasional phenomenon [22] and the mechanism of resistance is unresolved [5].

Ligand-gated cation channels

Nicotinic acetylcholine receptors (nAChRs) are widely expressed in the nematode nervous system, both at the neuromuscular junction (on the muscle cells) and on

the neurones themselves [23, 24]. They are targeted by several anthelmintics, including levamisole [25], pyrantel [26], oxantel [27], morantel, paraherquamide [28], and the recently announced AADs [6]. Most of these compounds are agonists at the neuromuscular receptor, causing a spastic paralysis of the worm, though paraherquamide is an antagonist. It is clear that not all of these compounds act at the same receptor and it is also clear that nematodes possess multiple forms of nAChR. Richard Martin and colleagues have identified at least three pharmacological and physiological subtypes at the body wall neuromuscular junction and several others are likely to exist in the pharynx, head muscle, and central ganglia [29, 30]. The AADs exploit this receptor diversity, probably acting at a distinct group of nicotinic receptors in *Caenorhabditis elegans* and the sheep gastrointestinal nematode *Haemonchus contortus* [6]. These receptors are likely to contain subunits such as DES-2 and ACR-23 that are expressed on pharyngeal muscle, ventral cord interneurones, and sensory neurones [31]. These subunits seem to be unique to nematodes; and this may explain the lack of AAD toxicity to mammals, insects, and other invertebrates.

The cyclo-octadepsipeptides such as PF1022A or emodepside interact with a G protein-coupled, seven transmembrane domain receptor named latrophilin 1 in *C. elegans* or HC110-R in *H. contortus* [19]. Emodepside was found to specifically bind to the extracellular N-terminal part of HC110-R. This receptor can be activated by the black widow spider venom latrotoxin, resulting in calcium influx into eukaryotic cells recombinantly expressing HC110-R. Preincubation of these cells in cyclo-octadepsipeptide solutions postpones and minimizes the latrotoxin effect in a concentration-dependant way, indicating that emodepside directly acts through HC110-R. Full efficacy of emodepside observed against benzimidazole-, imidazothiazole-, and ivermectin-resistant populations of nematodes from sheep and cattle further confirmed that this drug class acts by a new mode of action [32]. *In vitro* mutagenesis in *C. elegans*, leading to complete emodepside resistance, revealed that a large-conductance calcium-activated voltage-gated potassium channel, named SLO-1, is also essential for the mode of action [20]. SLO-1 mutants showed resistance in pharyngeal pumping as well as in locomotion assays applied to investigate the emodepside effects. In contrast, only pharyngeal pumping activity was observed to be resistant to emodepside in LAT-1 mutants, while body movement was still normally inhibited. This indicates that more than one endogenous cyclo-octadepsipeptide receptor exists. The forward genetics approach performed by Guest *et al.* [20] showed that only SLO-1 mutants were generated, which argues for a signalling of emodepside directly through a SLO-1 pathway, presumably at the postsynaptic membranes at neuromuscular junctions in the body wall musculature and at presynaptic sites in the pharynx [33]. However, further data are needed to fully understand the relevance of the currently known components in the emodepside action pathway, in particular in parasitic nematodes.

Ligand-gated anion channels

Nematodes possess an abundance of ligand-gated anion channels, with a greater variety of ligands than are found in vertebrates. γ-Amino butyric acid (GABA) is common to both phyla, but fulfils quite different roles: in nematodes it acts as an

inhibitory transmitter at the neuromuscular junction and is also present in some other neurones [34, 35]. Its receptors are found on muscle cells, but are not currently major anthelmintic targets, though piperazine is a GABA receptor agonist and causes a flaccid paralysis [36]. By far the most important group of compounds that act at ligand-gated chloride channels are the macrocylic lactones, which include ivermectin, moxidectin, doramectin, selamectin, eprinomectin, and milbemycin oxime. All of those compounds are very hydrophobic and have an unusual mode of action: though they do not bind to the normal agonist site, they irreversibly open the channels, leading to a permanent hyperpolarization of the cells and paralysis [37]. They bind to, and activate, a wide range of ligand-gated anion channels, but in nematodes their most important targets are the glutamate-gated chloride channels (GluCl), which are expressed widely in the nervous system and on pharyngeal muscle [38–43].

Specific Resistance Mechanisms

In this context, we define specific resistance mechanisms as those that confer resistance to only one class of drugs, with little or no effect on other classes. These forms of resistance might be expected to be caused by changes in the drug target site or in specific activating enzymes, rather than by an increase in the expression of detoxifying enzymes, like P450, or efflux pumps, like the ABC pumps in the plasma membrane. However, in some cases such an attempt at classifying resistance mechanisms may break down, as discussed below for the example of β-tubulin polymorphisms and macrocyclic lactone resistance.

C. elegans

The generation of drug-resistant strains of *C. elegans* and the genetic and molecular characterization of the mutations responsible for this resistance was an extremely productive and informative strategy for studying the mechanisms of action of all the current anthelmintic classes (Table 2.1). As such, these studies provide a wealth of important information and many valuable resources, but their relevance to the resistance mechanisms that develop in the clinical or field situations is more questionable. There are several reasons for this. Often, the drug concentrations used in the selection of the resistant mutants are rather high, in excess of anything that parasites would ever be exposed to. As one example, the concentrations of ivermectin used to study the genetics of resistance in *C. elegans* were up to 10 μg/ml, as opposed to the wild-type EC_{27} of ~1 ng/ml [42]. However, the same study also illustrates one great strength of the *C. elegans* research, which is its capacity to dissect out complex genetic interactions resulting from the occurrence of multiple drug targets. In this case, mutations in three independent genes were required for high-level resistance, reflecting at least three different forms of the GluCl target found in

Table 2.1 Drug resistance genes identified in *C. elegans*.

Drug	Gene	Gene product	References
Benzimidazole	*ben-1*	β-Tubulin	[47]
Levamisole	*lev-1*	Nicotinic acetylcholine receptor α-subunit	[25]
	lev-8/acr-13	Nicotinic acetylcholine receptor α-subunit	[48]
	lev-9	Not yet published	
	lev-10	Transmembrane protein required for nAChR clustering	[49]
	lev-11	Tropomyosin	[50]
	unc-22	Twitchin	[51]
	unc-29	Nicotinic acetylcholine receptor subunit	[25]
	unc-38	Nicotinic acetylcholine receptor subunit	[25]
	unc-63	Nicotinic acetylcholine receptor subunit	[52]
Ivermectin	*avr-14*	Glutamate-gated chloride channel subunit	[42, 53]
	avr-15	Glutamate-gated chloride channel subunit	[39, 41]
	glc-1	Glutamate-gated chloride channel subunit	[38]
Emodepside	*lat-1*	Latrophilin	[54]
	slo-1	Potassium channel	[20]
AAD	*acr-23*	α-7-Like subunit of the nicotinic acetylcholine receptor	[6]

the worm. Mutations in any one target did not prevent the drug from acting at any of the other two. The mutagenesis protocols used in *C. elegans* tend to lead to deletions, or loss-of-function mutations; and these may not always reflect the sequence variability (on which drug selection acts) present in parasite populations. Mutant strains of *C. elegans* can be easily maintained in the laboratory, even with severe defects or fitness costs that could never survive in the field; and so the mechanisms of resistance may reflect a spectrum of possibilities rather than those most likely to occur. It may also be dangerous to assume that the genetic composition of a parasitic species is the same as *C. elegans*. As an example, the levamisole-resistance *C. elegans* genes *lev-1* and *lev-8* (*acr-8*) are not present in parasites like *Brugia malayi* and *Trichinella spiralis* [44].

Studies on specific anthelmintic-resistance mechanisms in *C. elegans* have therefore been invaluable in elucidating drug targets and mechanisms of action, which are generally conserved across nematode species, but have so far been less useful in revealing the resistance mechanisms likely to be found in the clinic or the field. An additional contribution to parasitology from *C. elegans* is the use of the model organism as an expression system for parasite genes; and such studies have been applied to drug resistance genes. For example, the benzimidazole resistance-associated polymorphisms in the *H. contortus* β-tubulin isotype 1 also confers drug resistance onto transgenic *C. elegans* [45]; and the GluCl gene products from the same parasite can also be successfully expressed in the model worm [46].

Parasitic Nematodes

Although there are numerous reports on the isolation of drug-resistant parasitic nematodes, or their generation under controlled conditions, in very few cases have any genetic or molecular mechanisms of resistance been identified. Probably the only major anthelmintic class for which a resistance mechanism has been unequivocally shown is the benzimidazoles, resistance against which is associated with changes in β-tubulin [12]. The genetic and biochemical evidence for the involvement of alterations in β-tubulin in benzimidazole resistance is overwhelming, though it does seem that several different changes may be involved (at amino acids 167, 198, 200) [13, 14, 55–58] and that their relative importance may vary between parasite species and possibly between isolates.

The most common single nucleotide polymorphism (SNP) that causes benzimidazole resistance in *Haemonchus contortus* is the change at codon 200 (TTC to TAC) in β-tubulin [4, 15, 16, 58]. This change leads to the expression of tyrosine instead of phenylalanine (F200Y) and is interesting because tyrosine is the amino acid residue present at the homologous position in mammalian tubulin. This change causes a reduction in the affinity of binding of the drug to recombinant protein [17] and, as described above, can confer resistance onto transgenic *C. elegans*. However, in other nematodes(e.g., *Cyathostomins*) the same SNP at codon 167 (Phe to Tyr) may be common [13, 15]. The Glu198Ala SNP may be uncommon and some evidence suggests that the Phe200Tyr, Phe200Tyr or Glu198Ala SNPs do not occur in the same allele [16]. The 200Tyr β-tubulin commonly found in benzimidazole resistant nematodes is the same codon as occurs in mammalian hosts, as mentioned above. So there is little likelihood that molecules which bind to the resistant worms' β-tubulin would be nontoxic in the host; and no obvious means exists to overcome this target site resistance while still acting on the benzimidazole binding site.

For the other anthelmintics, we have much less definite information on specific resistance mechanisms. Resistance to AAD in a laboratory-generated resistant strain of *H. contortus* is associated with a loss of expression of two nAChR subunits [6], but since these compounds are not yet commercially available, there are no isolates available to confirm that this is relevant in the field. No emodepside-resistant parasites have been reported, so the involvement of *lat-1* and *slo-1* homologs in resistant to this drug cannot yet be confirmed. Even where there are abundant resistant parasites to study, such as for the macrocyclic lactones and levamisole, it has been problematic to demonstrate the involvement of any of the known target genes. The GluCl genes *avr-14* and GluClα have been shown to be under selection pressure in macrocyclic lactone(s)-resistant isolates of *H. contortus* [59], but no specific resistance-associated sequence changes have been identified. The *avr-14* of *Cooperia oncophora* does show such a change between a resistant and a susceptible isolate, the L256F polymorphism that *in vitro* causes a reduction in sensitivity to ivermectin [60], but this polymorphism has yet not been detected in any other species or isolates of *C. oncophora* (Samson-Himmelstjerna and Wolstenholme, unpublished data). The situation may be complicated if, as suggested, there are multiple forms of resistance and if these differ between laboratory-generated and field isolates [61] and/or if

resistance is polygenic [62]. Additional complications may arise because of the multiple macrocyclic lactone(s)-targets present in parasitic nematodes and the likelihood that their relative importance may vary between species. Inhibition of locomotion or feeding may be most important for gastrointestinal nematodes, but less so for tissue-dwelling species such as the filaria, where the reduction in fecundity seems to be the major effect.

A major complicating issue may be that macrocyclic lactone(s) resistance has also been suggested to select for the β-tubulin F200Y polymorphism associated with benzimidazole resistance [16, 63]. At present, we have no mechanistic explanation for this and its contribution to the resistance phenotype is unclear. However, it could somewhat complicate the interpretation of studies that use molecular tools to monitor benzimidazole resistance.

For levamisole, there is pharmacological evidence for a reduction, or absence, of the drug-sensitive "L"-form of nAChR in resistant *Oesophagostumum dentatum* [64], but no further molecular or genetic data. Overall, the picture for specific drug resistance mechanisms in parasitic nematodes is frustrating; and only in the case of the benzimidazoles has a convincing link been made.

Nonspecific Resistance Mechanisms

Nonspecific resistance mechanisms can include drug transport mechanisms with relatively low specificity, usually involving ABC transport proteins, or drug metabolism such as oxidation by cytochrome P450. There is little evidence that oxidative drug metabolism (e.g., involving cytochrome P450) is very active in parasitic nematodes [65] and there is no evidence that drug metabolism plays a significant role in resistance to existing anthelmintic drugs.

However, drug efflux mechanisms may play a significant role in resistance to some anthelmintics. In particular, there is strong evidence that ABC transporters, such as P-glycoproteins, are involved in resistance to macrocyclic lactones. Initial evidence that ivermectin is an excellent substrate for efflux mechanisms by mammalian P-glycoprotein [66] provided a rationale for parasitologists to investigate whether ivermectin-resistant parasites used similar mechanisms to protect themselves against macrocyclic lactone anthelmintics.

Data gathered over the past few years seems to confirm this hypothesis [75]. In 1998, it was demonstrated for the first time that ivermectin-selected *H. contortus* showed higher expression of PgpA, a P-glycoprotein found in *H. contortus*, compared with a parental unselected strain [67]. Furthermore, the use of the MDR-reversing agents verapamil and CL347 099 increased the efficacy of ivermectin and moxidectin against macrocyclic lactone-resistant *H. contortus* strains in jirds [68]. Initial studies focused on a single P-glycoprotein, PgpA, in ivermectin-resistant *H. contortus*, for which a computational molecular model has been published [69]. Recently, it was reported that ivermectin and moxidectin treatments select for a constitutive or

inducible overexpression of at least five P-glycoproteins in adult *H. contortus* worms (PgpA, -B, -C, -D, -E) [62]. The constitutive overexpression could be a result of mutations in the regulatory sequences of the Pgp genes, which are frequently located in the promoter region or introns. The mechanism behind the inducible overexpression is still not understood. However, it is of interest thatP-glycoprotein expression in mammalian cells can be upregulated by the induction of oxidative stress [70].

It has also been shown that, in *Onchocerca volvulus*, multiple ABC transporter genes, including P-glycoproteins [71, 72] and a half-sized ABC transporter [73, 74] are selected by ivermectin [75]. However, to date expression studies have not been conducted in ivermectin-resistant *O. volvulus*.

In contrast to the situation in mammals, nematodes have multiple P-glycoprotein genes. Fifteen P-glycoprotein encoding genes have been identified in the genome of *Caenorhabditis elegans*, including one pseudogene. The reason for this multitude of ABC transporters in nematodes is not known, but it was recently suggested that they might be essential to protect different neurons in the body of the nematodes from toxins [62].

Another interesting aspect about the interaction of macrocyclic lactones with P-glycoproteins is the observation that different macrocyclic lactones appear to have different P-glycoprotein efflux potential, at least with the mammalian P-glycoprotein mdr1; with ivermectin having an IC_{50} approximately 10× lower than moxidectin [76]. The efflux activity of mammalian mdr1 in the host blood–brain barrier appears to be critically important for preventing central nervous system toxicity by ivermectin [77–79], and perhaps some other avermectins. Although the interaction of ivermectin with P-glycoprotein is strong, it also interacts with mammalian multidrug resistance proteins MRP1, 2, and 3 [80], indicating that the efflux of MLs in the mammalian host, and possibly in the parasite, is under the control of more than one class of ABC transporter.

While benzimidazole resistance seems to be largely due to one of the allelic changes in β-tubulin, described above, which cause loss of high affinity benzimidazole binding, there is mounting evidence that efflux mechanisms involving P-glycoproteins or other ABC transporters may contribute to benzimidazole resistance [81–83].

In the search for new antiparasitic drugs, it could be important to assess the ability of drug candidates to be transported by ABC transporters, in order to reduce the possibility that the target parasites could rapidly develop efflux mechanisms of drug resistance against the new entity, and also because of the role of ABC transporters in determining the pharmacokinetic behavior and possible toxicological properties of a drug [62].

Contribution to Drug Discovery

Understanding the mechanisms of resistance to any drug raises the prospect that the resistance may be reversed or countered by the development of new agents. For the

specific mechanisms of resistance discussed in this chapter, it may be difficult to see how this might be accomplished; for example, benzimidazole resistance-associated mutations tend to make the parasite β-tubulin sequence more like that of the mammalian hosts. However, in the case of levamisole, it could be argued that our appreciation of the multiplicity of nicotinic acetylcholine receptors present in nematodes has led to the recent progress in developing novel agents (AAD and the paraherquamide derivatives), effective against levamisole-resistant parasites, which target levamisole-insensitive receptors.

Conclusions

Anthelmintic resistance clearly has major scientific, technical and, last but not least, economical implications on the future of drug discovery. Scientifically, the understanding of mechanisms of anthelmintic resistance can contribute to elucidate the mode of action of anthelmintics. This leads to new drug target identification and characterization enabling, for example, the development of specific drug screening assays. Testing resistant worm populations with new drug candidates provides strong indications concerning similarities or differences in the mode of action of different compounds from the same chemical class and from different classes. Technically, we may be forced to more seriously consider and develop improved strategies of worm control with the key focus on sustainability of present and new drug classes. Treatment regimes like targeted selected treatment (TST) or prevention treatment at times without refugia may become the main strategies within future worm management due to the increased advent of multi drug resistant worm populations. Moreover, combinations of drugs with a similar spectrum of efficacy but a different mechanism of resistance will be of increasing importance on the veterinary pharmaceutical market of countries with severe anthelmintic resistance. New drugs may be considered both alone and for combinations with existing drugs, for example, to complement their spectrum of efficacy but also to postpone the development of resistance by redundant killing effects [84]. Furthermore, synergistic efficacies could be associated with combinations due to additional resistance-preventing effects.

Economically, the event of increasing anthelmintic resistance in cattle, horses, and possibly even in pigs and pets will certainly lead to reinforced efforts to find new drugs by the pharmaceutical industry. Together, the anthelmintics and endectocides were worth around $2.5 billion in 2006, with the largest segments being the avermectins (>30%), followed by the benzimidazoles (<10%) and imidathiazoles (<10%; P.M. Selzer, personal communication). In an essentially saturated market and with estimated total research and development costs of more than €200 million for a new anthelmintic compound, the stimulus for strategic investments in this direction is directly dependent on the advent of resistance against the established drug classes.

It is apparent that already an enormous body of scientific information has evolved from studying anthelmintic-resistant worm populations. This area of research will

receive additional impetus by the outcomes of research in related fields such as the various helminth genome sequencing projects, proteomics, and protein biochemistry, as well as electrophysiology and pharmacology. We still need to learn in detail what the specific and nonspecific mechanisms of anthelmintic resistance are; and this most probably has to be done not only separately for every drug class but presumably also in a worm species-specific manner.

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3
Drug Discovery for Neglected Diseases: View of A Public–Private Partnership

Rob Don and Eric Chatelain*

Abstract

In answer to the lack of modern and effective drugs for diseases such as human African trypanosomiasis (HAT; sleeping sickness) and Chagas' disease which present no financial viability for the pharmaceutical industry, new models of drug discovery have been developed. Public–private partnerships (PPPs) or product development partnerships (PDPs) aim to combine the skills and research capacity of academia, the pharmaceutical industry, and contract researchers to create focused research consortia which address all aspects of drug discovery. These consortia emulate the project teams within the pharmaceutical and biotechnology industry and include identification and screening of libraries, medicinal chemistry, pharmacology, and pharmacodynamics. The Drugs for Neglected Diseases initiative (DND*i*) has adopted a model closely related to that of a virtual biotechnology company for identifying and optimizing drug leads. This chapter outlines the application of this model to the development of drug candidates for the kinetoplastid infections of HAT, Chagas' disease, and leishmaniasis.

Introduction

During the past century, chemical and pharmaceutical industries made tremendous advances in the development of new therapeutics to treat many of the diseases which inflict humankind. This drug discovery and development process, which encompasses design of new drug candidates and clinical testing, has always been an expensive exercise, with costs continuing to escalate [1]. To a large extent, this cost has been borne by the consumers either directly or through taxes and insurance-based subsidies available only in wealthy countries. Those diseases which predominantly affect inhabitants of poorer nations, are of no military or strategic interest to wealthy countries and are not supported by markets or patients organizations capable of attracting the attention of politicians, have fallen below the radar of modern drug

discovery. Moreover those afflicted are unable to bear the cost of development. These diseases have therefore been forgotten and often labeled as neglected diseases or, perhaps more appropriately, "diseases of the neglected." Examples of neglected diseases are malaria, dengue fever, tuberculosis (TB), human African trypanosomiasis (HAT), leishmaniasis, Chagas' disease, and buruli ulcer. There is a considerable global effort to bring awareness to these diseases and redress such inequity.

> "*Our greatest concern must always rest with the disadvantaged and vulnerable groups. These groups are often hidden, live in remote rural areas or shantytowns and have little political voice*"
>
> *Dr Margaret Chan – WHO Director General, 2007*

Unfortunately, the tools to treat these diseases fall woefully short of what many in wealthy nations would expect in a modern pharmacopoeia. For example, first-line therapy for stage 2 human African trypanosomiasis (HAT; sleeping sickness) in many regions is still melarsoprol, an arsenic-based drug developed at the turn of the twentieth century which causes fatal encephalopathy in up to 5% of those treated [2, 3].

Another example: long-term treatment with genotoxic drugs is the only choice for Chagas' disease in which a significant proportion of patients are children with a median survival measured in decades [4, 5]. There is an urgent need to bring the medicine chest for neglected diseases up to date for the sake of patients' wellbeing. To do so requires the development of different strategies to mobilize drug discovery from the "user pays" model in wealthy countries to one which can be sustained for those unable to pay.

Public–Private Partnerships

Over the past decade, a number of new ventures classified under the broad term of public–private partnerships (PPPs) have emerged to address the need for new drugs to treat neglected diseases [6]. Initially these were formed to focus on specific diseases and examples include the Medicines for Malaria Venture (MMV), Global Alliance for TB Drug Development (TB Alliance), the International Aids Vaccine Initiative (IAVI), and the Malaria Vaccine Initiative (MVI). More recently, ventures with a broader focus (efforts not concentrating on a single disease) have emerged such as the Institute for One World Health (iOWH) and the Drugs for Neglected Diseases initiative (DND*i*), and definitions of PPPs have expanded to include not-for-profit pharmaceutical companies and product development partnerships (PDPs) – perhaps a reflection of their different strategies to achieve a common goal, that is, to provide drugs for neglected patients [7].

DND*i* was formed in 2003 with the aim of developing new drugs for a group of "most neglected diseases," the kinetoplastid diseases. In the first instance theses diseases include a group of infectious parasitic diseases: human African trypanasomiasis, visceral leishmaniasis (VL), and Chagas' disease.

Human African Trypanosomiasis (HAT)

HAT, known as sleeping sickness, is caused by two subspecies of *Trypanosoma* parasites which are transmitted to humans by tsetse flies. Sleeping sickness, which WHO estimates to infect 50 000 to 70 000 people and puts 50 million at risk, occurs only in subSaharan Africa [8]. The disease takes one of two forms, depending on the parasite subspecies (either *T. b. gambiense* or *T. b. rhodesiense*). Sleeping sickness has two stages. The early stage entails bouts of fever, headaches, pain in the joints, and itching. The second, known as the neurological phase, begins when the parasite crosses the blood–brain barrier and invades the central nervous system. Without treatment, the disease is fatal. Currently available treatments for HAT – melarsoprol, eflornithine, pentamadine, and suramin – are few and limited due to age, high toxicity, and lost efficacy in several regions. Treatment is stage-specific, with more toxic and more difficult-to-administer treatments (melarsoprol, eflornithine) for stage 2 disease. Few projects for improved treatments are currently in clinical development and none has the potential to dramatically change either the treatment or control options for this disease. With regard to treatment, most drugs are old, difficult to administer in resource-limiting conditions and by no means always successful [9, 10].

Visceral Leishmaniasis

Transmitted by the sandfly, the protozoan parasite *Leishmania* causes three different forms of disease, of which visceral leishmaniasis (VL) is the most severe. Leishmaniasis affects over 12 million people and puts over 350 million people at risk in 88 countries. Fatal if left untreated, VL (also known as black fever or kala-azar in India) persists today in poor, remote, and sometimes politically unstable areas, in countries where patients have little access to preventive measures and affordable drugs. A significant proportion of clinical cases occurs in children. Approximately 500 000 new cases are reported to occur each year, though it is estimated that only 30% of cases are reported. VL is characterized by prolonged fever, enlarged spleen and liver, substantial weight loss, and progressive anemia and is complicated by co-infection with other infectious diseases, such as HIV, malaria, or pneumonia [10–12].

Chagas' Disease

Chagas' disease is another human form of trypanosomiasis (human American trypanosomiasis) and occurs almost exclusively in the Americas where an estimated eight to 11 million people are infected. Transmitted to humans by a triatomine insect containing the parasite *Trypanosoma cruzi*, the disease is contracted through the bite of insects widely known as the kissing bug. There are three stages of the disease: acute, indeterminate, and chronic. In the acute form (in which 5% of children die), Chagas' disease manifests generally as fever, malaise, facial edema, generalized lymphadenopathy, and hepatosplenomegaly. The acute illness often spontaneously

resolves itself in four to six weeks, at which time patients enter an asymptomatic, indeterminate phase, which can last from 10 years onwards. The chronic stage of Chagas' disease develops in 10–30% of infected persons and most commonly affects the heart. Death usually results from cardiac arrhythmia or congestive heart failure. The two current treatments, benznidazole (which requires 60 days of treatment in acute infections and is only effective in 50% of cases) and nifurtimox (primarily acute and early indeterminate stages of the disease) are very limited. There are no treatments for indeterminate and chronic stages of the disease [10, 13].

DND*i*'s Partners and Strategy

DND*i*'s founding partners are Médecins Sans Frontieres, the Oswaldo Cruz Foundation for Brazil, the Indian Council for Medical Research, the Kenya Medical Research Institute, the Ministry of Health in Malaysia, the Pasteur Institute in France, and the Special Programme for Research and Training in Tropical Diseases (TDR). Their vision was the creation of a drug discovery and development entity using the assets of organizations spread across the world to respond to the dire need of safe, affordable, easy-to-use, and efficacious treatments for neglected patients [14]. Since its inception, DND*i* has invested resources in preclinical and clinical development as well as drug discovery programs with the intent to bring "low-hanging fruits" to patients in the shortest possible timeframe. This approach has been so far implemented with two products now registered for treatment of malaria: (i) fixed-dose artesunate-amodiaquine, "AS/AQ" (www.actwithasaq.org), and (ii) fixed-dose artesunate-mefloquine, "AS/MQ" (www.actwithasmq.org), programs in phase II and III clinical trials for visceral leishmaniasis and sleeping sickness, and a strong network of clinical researchers and trial sites in disease-endemic regions. Nonetheless, the organization remains acutely aware of the high attrition rate associated with drug discovery and development and strives to maintain a full pipeline, which includes development of innovative new drugs through longer-term lead optimization programs [15].

Lead Optimization at DND*i*: Building the Pipeline

During the five years since its inception, DND*i*'s model of drug discovery has evolved from funding research projects in response to "letters of interest" to building a virtual and fully integrated, patient-need driven, not-for-profit, R&D organization. The initial model (i.e., responding to "letters of interest") which very successfully reviewed the research landscape for neglected diseases also led to the risk of DND*i* being perceived as a research funding agency rather than a drug discovery organization. As such, if initial research projects were successful, an expectation existed for DND*i* to increase

commitment and expand the projects – a natural progression based on the model of competitive research funding. Keeping the "letter of interest" approach would have required:

- Expanding a team which had commitment and expertise frequently limited to one particular molecular scaffold and/or target;
- Building the capacity in teams which did not span all of the research disciplines necessary for drug discovery;
- Losing the ability to prioritize projects because of a need to fund projects on a medium term basis at research institutions and therefore commit limited resources on a "first come/first served" basis.

In building such lead optimization teams, considerable expense and time would be needed during the expansion phase and, if the team was focused on a particular scaffold or target, considerable time and expense would also be required to wind the project down if that target proved not amenable to lead optimization. To acknowledge the high rate of attrition in drug discovery and counter the probability of disbanding research programs because of drug candidate failure, DND*i* has adopted the approach of contracting lead optimization teams independent from specific targets or scaffolds. In doing so, DND*i* can feed each team with hits and leads which are identified from a range of different sources. Scaffolds which prove not to be amenable to optimization can also be "killed" without disrupting the research integrity of the dedicated lead optimization team.

To maintain the capacity of optimizing one lead series at all times, each team is comprised of 5–6 chemists, 2–3 pharmacologists, and a dedicated screening facility to assess potency and efficacy with guaranteed infrastructure to support medicinal chemistry, *in vitro* and *in vivo* distribution–metabolism–pharmacokinetic (DMPK), toxicology (Tox.), and efficacy studies. In addition, DND*i* works with consultants who are expert in different disciplines to provide ongoing critical review of the programs. The programs are also regularly reviewed by the Scientific Advisory Committee (SAC) of DND*i*. The SAC is a group of 15 respected scientists who volunteer their time to assess and guide DND*i* scientific activities.

To produce new drug candidate as efficiently as possible, it is very important that all players are focused on the lead optimization program and not distracted by other research efforts. To that end:

- Once a lead is introduced into a lead optimization program, it enters a critical path [16] which promises development through to patient access unless the compound series fails because structural liabilities prevent optimization as a drug candidate;
- Sufficient resources are allocated to guarantee the rapid turnaround of data necessary to support the medicinal chemistry effort;
- A research group associated with the program is encouraged to pursue more discovery research associated with the disease so that the lead optimization (LO) programs are constantly fed with promising leads. This implies that a separate

group of staff conducts the supporting assays for lead optimization and commits 100% of its time to the medicinal chemistry effort.

To date, DND*i* has brought together three LO teams, two for HAT, and one for VL. DND*i* has chosen to work with companies and partners with a commitment to research for neglected diseases at the senior management and CEO level to ensure that research goals are aligned. The researchers are funded as full "fee for service" contractors and make no claim to any intellectual property generated during the lead optimization programs. As such, DND*i* remains free to manage the intellectual property in a manner the organization deems to be most appropriate to guarantee access for those suffering from neglected diseases.

Lead Optimization at DND*i*: Supporting the Pipeline

Target Product Profiles

DND*i* is a needs-driven organization, that is, the needs of patients suffering from neglected diseases are the driving force behind all drug discovery and development programs. DND*i* partners work with patients in disease endemic countries and DND*i* has actively participated in bringing together stakeholders like physicians, local disease control programs, regulators, and patient representatives, as well as local and global public health bodies to gauge the true needs of patients. It is through consultation with expert committees that DND*i* is able to draft target product profiles (TPPs), which define effective therapies for patients in normal field conditions.

The TPP is an organized list that prioritizes key features of the drug and guides all players to work with the same end in mind [17]. It lists both ideal and acceptable values for each feature. On a strong note of caution, one should exercise care not to lower development standards and use the acceptable values as the TPP. They should be viewed as the lowest acceptable value for a particular feature in isolation. If this value becomes part of the TPP then other values originally listed as acceptable may be compromised and the TPP should be revised. For example, if parenteral administration instead of oral is deemed acceptable then a previously acceptable treatment duration of two months may no longer be tenable.

The TPP (see Table 3.1 for an example) is the foundation of all drug discovery management tools designed to support lead optimization programs.

Discovery Manuals

To direct a lead optimization program, a drug discovery manual is drafted which defines key decision points during the preclinical drug discovery process and provides objective measures for making those decisions with reference to potency,

Table 3.1 Example of a TPP for human African trypanosomiasis.

Desirable	**Acceptable: improvement to current standard treatment**
Effective against stage 1 and 2	Effective against stage 2
Broad spectrum (*T. gambiense, T. rhodesiense*)	Efficacy against *T. gambiense* only
Clinical efficacy >95% at 18 mo follow up	Clinical efficacy no worse than current treatment
Effective in melarsoprol refractory patients	
<0.1% drug related mortality	1% drug related mortality
Safe during pregnancy and for lactating women	
Formulation adapted to adults and children	
No monitoring for AEs	Weekly simple laboratory testing (field testing)
<7 d p.o. once daily (DOT)	<20 d p.o. (DOT)
<7 ds i.m. once daily	<20 d i.m.
	<5 d i.v. if no toxicity
Stability in zone 4 for >3 yr	Stability in zone 4 for >12 mo
Cidal	
multitarget	Unique target (but no uptake via P2 transporter only)
< €30/course (only drug cost)	< €100/course
	< €200/course; ok if very good on other criteria

This TPP is revised on a regular basis.
Considering that some 20 000 to 50 000 patients per year might require treatment, it is still expected that donor agencies rather than the patients themselves take care of these costs.

physicochemical properties, and *in vitro* and *in vivo* DMPK/Tox. The major decision points are listed below and shown in Figure 3.1:

- Hit Selection – The decision to proceed to *in vivo* studies and hit expansion based on identification of a single compound from library screening or literature search.
- Lead Selection – The decision to proceed with chemical optimization based on review of a series of chemical analogs of the "Hit."
- Optimized Lead Selection – The decision to proceed to regulatory preclinical toxicology, drug metabolism and pharmacokinetic (DMPK)/Tox., and "good manufacturing practice" pharmaceutical development of one compound or a small number of compounds from the same chemical class.
- Drug Candidate Selection – The decision to proceed to clinical evaluation of one compound or a small number of compounds from the same chemical class.

In addition, key activities and partners are listed for each stage of drug discovery to ensure that sufficient resources have been allocated to the program and that research progresses in the shortest timeframe as activities are conducted in parallel wherever possible. At each decision point, the information may be sufficient to result

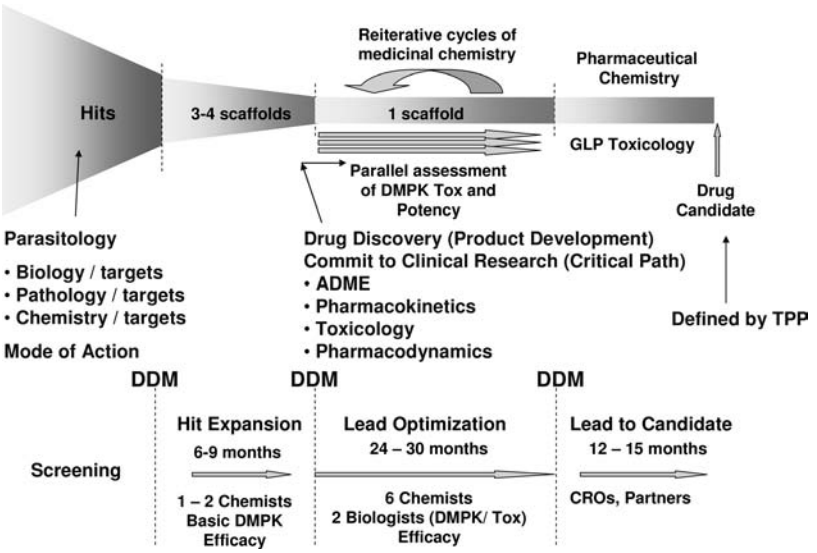

Figure 3.1 DNDi's drug discovery process with decision-making tools and key decision points (DDM: drug discovery manual; TPP: target product profile).

in a "go/no go" decision. In other instances, it may highlight additional research required before fully committing to the next stage of drug discovery.

The key activities for each decision point and the expected outcomes/values for these activities are defined to guarantee that continued development will address the expectations defined in the TPP.

Decision Matrices

The decision matrix is an operational tool which is designed to support the drug discovery manual. In addition to the key assays specified for the decision points, simpler surrogate assays are listed to enable more rapid screens of molecules which are synthesized as part of medicinal chemistry programs. For instance, membrane permeability may be used to predict bioavailability or ability to cross the blood–brain barrier. As the program progresses, more complex assays with greater predictive value should be used. Ideal and acceptable values together with data for comparator drugs are included in the decision matrix to facilitate assessment of new compounds with respect to the TPP and current drugs. A simple spreadsheet in which values for each new compound are entered allows for ready comparison with ideal and acceptable values. By color coding the cells, based on the value entered (green for ideal, orange for acceptable, red for unacceptable), it is possible to rapidly assess progress during the synthesis of a particular chemical series. In addition, it is recommended that representatives from major compound classes with defined biological activity should be regularly tested in key assays to assess the true predictive value of the surrogate assays.

Data Management

Regular and spontaneous communication between all members of a research team is essential for efficient progression of a lead optimization program. In most cases, not all members are located at the same site. For instance, it is rare that efficacy studies for tropical infectious diseases can be sourced to commercial contract researchers carrying out medicinal chemistry. Such studies are usually contracted to a University research group. To provide all participants in the research exercise with ready access to data as generated, DND*i* uses a proprietary web-based data management system. All researchers enter data in templates which have been formatted to suit the data they generate, be it chemical or biological. The data is structure and substructure-searchable and every researcher can review all data within the context of their specific expertise. A web-linked based portal has the capacity to store files in a variety of formats and is used for circulation of reports and for storing complex supporting data and background information such as papers and patents.

Lead Optimization at DND*i*: Feeding the Pipeline

Access to an adequate chemical diversity of hits and leads is essential to guarantee success for each lead optimization team. By generating such diversity, new chemical series can be introduced to the lead optimization process when scaffolds fail due to the natural attrition associated with drug discovery. In line with DND*i*'s approach to develop compounds as far downstream as possible ("low-hanging fruits"), DND*i* identifies small series of compounds in which structure activity relationships may already be available or may be readily discerned rather than large libraries of compounds for early stage hits.

Compounds and compound series may be focused around inhibitors of a specific target or a molecular scaffold known to be associated with antiparasitic activity. They are sourced from pharmaceutical companies, biotechnology companies, and academic institutes. To prevent diluting the effort of the lead optimization teams, DND*i* aims to support hit to lead chemistry with the partners who provide access to their compound libraries when possible. To support the constant feeding of hits in the LO process, DND*i* also sponsors a network of natural product screening centers worldwide. Natural product screening is conducted with the goal of identifying hits and/or leads which can be subsequently optimized as new chemical entities.

Negotiation of terms for access to intellectual property associated with the libraries is done on a case-by-case basis. It is DND*i*'s policy to disseminate intellectual property associated with neglected diseases research as widely as possible. Nonetheless, DND*i* remains pragmatic while negotiating and respects more stringent restrictions in return to access to new compounds which may lead to new therapies for treatment of neglected diseases, keeping in mind patient's interest. DND*i* is also aware of the risk

for contamination of intellectual property through sharing of information on closely related chemical series developed by different companies.

Conclusion

The product development partnership for neglected-disease drug development relies both on forging a strong relationship between industry and academia and on sharing knowledge which different partners often hold as proprietary. As well as the application of best research practices (e.g., TPPs, drug discovery manuals, critical paths), the building of trust and open communication are paramount to success. While the concept of PDP is still in its infancy, many productive alliances have been built, and the DND*i*-coordinated Fixed-Dose Artesunate-Containing Therapy (FACT) Project Consortium has developed and made available two new, improved treatments for malaria. In 2007, DND*i* registered its first product, the antimalarial "ASAQ," by applying its innovative R&D model, in collaboration with Sanofi-Aventis for the treatment of noncomplicated *Plasmodium falciparum* malaria. In 2008, DND*i* launched its second product for malaria, "AS/MQ," in Brazil using a similar model in partnership with a public institution (Farmanguinhos, Brazil). However, there remains a continuing need to advocate for increased awareness of the plight of those suffering from these neglected diseases. It is only by doing so that we can ensure that truly modern treatments – safe, efficacious, easy to administer – will become available for those afflicted by these "diseases of the neglected."

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4
Bioinformatics and Chemoinformatics: Key Technologies in the Drug Discovery Process

*Andreas Krasky, Andreas Rohwer, Richard J. Marhöfer, and Paul M. Selzer**

Abstract

The increasing resistance of many parasitic pathogens against current medical treatments requires the development of new effective drugs. A modern concept for the development of new antiparasitic drugs is the target-based approach. Using a combination of bio- and chemoinformatic techniques we show how the target-based approach can be supported by *in silico* methods, for example, comparative sequence analysis, homology modeling, and molecular docking. Starting from EST sequences, we identify potential target proteins suitable for the development of antiparasitic drugs and show how these targets can be used in the subsequent lead identification process. The ectoparasitic target SAM synthetase serves as an attractive example for illustrating the success of this combined *in silico* workflow. The data generated can be used for the development of specific inhibitors that attack the ectoparasitic SAM synthetase, thereby sparing the vertebrate enzyme.

The Need for New Drugs

Parasitic infectious diseases cause huge health problems and affect millions of people around the world. On the one hand parasites can directly impair their host organisms by sucking blood or depleting nutrients, on the other hand they can serve as vectors for dangerous pathogens like *Yersinia pestis*, the causative agent of bubonic plague, which is transmitted by the rat flea *Xenopsylla cheopis* [1]. Moreover, plant and animal parasites (e.g., *Heterodera glycines*, *Heterakis gallinarum*) provoke severe economic losses in agriculture and livestock farming [2, 3]. An inherent problem of parasitic infectious diseases is the increasing resistance of the parasites against current medical treatments, rendering some maladies almost completely untreatable. A parasite which has developed such strong resistance is *Haemonchus contortus*, the pathogenic agent of haemonchosis in sheep and goats [4, 5]. Living in

the abomasum and feeding on blood, it is one of the most pathogenic nematodes of ruminants which can eventually lead to death of the host organism.

Particularly devastating for both humans and livestock animals is the situation in poor countries in South America and Africa where protozoan diseases (e.g., malaria, trypanosomiasis) annually affect millions of people and farm animals, the latter bearing a vast economic impact [6, 7]. The main obstacle to treatment in these countries is that people cannot afford the necessary medication because the few effective drugs are by far too expensive [8]. These facts clearly show that new, reasonably priced, and resistance-breaking drugs are urgently needed [9, 10]. In the following sections we describe how new drugs are discovered using "wet bench" laboratory methods and how these methods are supported by a combination of bio- and chemoinformatics techniques.

The Drug Discovery Process

The systematic search for new drugs began in the second half of the nineteenth century. At that time, chemical substances were tested for biological activity in random-screening approaches, leading to successful drugs like Aspirin and many antiparasitics and antibiotics [11]. Today, the drug discovery process has become more rational, not least due to the decreasing success rate of the random-screening approach. A contemporary concept for the development of new anti-parasitic drugs is the target-based approach starting from a molecular point of attack, a target protein [12–14]. This approach can be divided into five main steps: target protein identification, target validation, lead identification, lead optimization, and finally biological testing of optimized lead structures [15]. A target protein has to meet several requirements: on the one hand it has to fulfill an essential function in the parasite, so that the inhibition or activation of the protein will result in damaging effects, on the other hand it should be different from its counterpart in the host organism – or even better completely absent – to minimize toxicity problems [16]. The latter applies to 1-deoxy-D-xylulose-5-phosphate reductoisomerase, a target of the antimalarial agent fosmidomycin (see Chapter 6, [17]). The target identification step usually involves conventional molecular biology techniques and high-throughput methods like functional genomics and bioinformatics. The second step, target validation, verifies whether an inhibition or activation of the target protein exerts an antiparasitic effect. This can be achieved by generating loss of function or gain of function mutants in animal models or by RNA interference [18]. In the case of endo- and ectoparasites such phenotype data can be deduced from the model organisms *Caenorhabditis elegans* and *Drosophila melanogaster* for which extensive studies have been carried out [19, 20]. In the subsequent lead identification phase, high-throughput screening (HTS) methods are used to identify small chemical compounds which are able to bind to the protein and thus interfere with its function. Apart from their

biological activity on the target protein, these compounds do not necessarily show all characteristics of a drug. Therefore, the lead structures are subjected to an optimization process in which chemical modification of the compounds is used to improve properties like bioavailability, selectivity, toxicity, stability, and potency [21]. Finally, the optimized lead compounds are tested in the target organism, the successful molecules being considered as candidates for subsequent drug development programs.

Bioinformatics and Chemoinformatics are Key Technologies in the Development of New Drugs

The rapidly growing amounts of biological and chemical data generated by the usage of high-throughput technologies require methods like bioinformatics and chemoinformatics, which are capable of turning data into knowledge. For instance, during the sequencing of the human genome, many billions of DNA nucleotides have been deciphered; and these cannot be handled manually, let alone be processed and analyzed manually [22]. The same is true for an example from chemistry, where hundreds of thousands of chemical structures are generated in each massive parallel synthesis approach [23]. It is obvious that, for dealing with such amounts of data, specialized technologies had to be developed for the storage and the analysis of the information. Improving high-throughput technologies like pyrosequencing, which is able to reveal the sequence of a bacterial genome within a few hours, make such analysis methods even more important in the future [24]. So these relatively new scientific disciplines emerged to key technologies being able to assist the target-based approach. The target identification process, for example, can be supported by bioinformatic comparison of biological sequence data, also known as genomic filtering [25]. These methods cover comparative sequence analysis, gene annotation, in-depth analysis of target proteins, microarray analysis, and so on [26]. In contrast, the lead identification and lead optimization steps can be substantially facilitated by chemoinformatic and molecular modeling techniques, comprising chemical property calculations, clustering of compounds, similarity searches, ligand docking, pharmacophore modeling, and the calculation of quantitative structure–activity relationships (QSAR) and ADMET properties [27]. One should keep in mind that chemoinformatic and molecular modeling methods are often regarded as separate scientific fields [11, 27], but reflecting our workflow we tend to combine these related techniques.

Acting together, bioinformatics and chemoinformatics can support and complement biological and chemical "wet bench" laboratory techniques, thereby extensively contributing to the drug discovery and vaccine development process (Figure 4.1).

Hence, the idea emerged to design a combined *in silico* workflow supporting the whole process from target identification to lead optimization, thus leading from sequences to potential drug candidates.

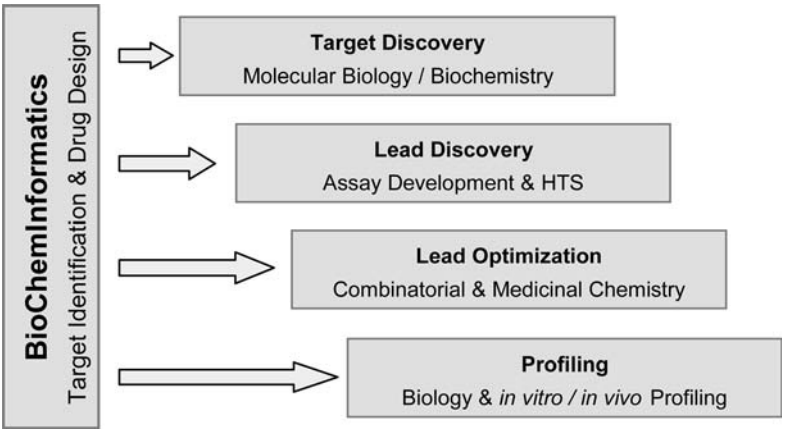

Figure 4.1 Drug discovery process. This diagram shows the whole drug discovery process in which the biological and chemical wet bench technologies are significantly supported by the *in silico* methods bio- and chemoinformatics. Depending on existing information, the process can also start at later steps of the workflow or the direction can be reversed in order to elucidate the mode of action of drugs.

Combining Bio- and Chemoinformatics – A Case Study

Starting from expressed sequence tags (ESTs) which are derived from mRNA and represent a valuable source of sequence data as long as no complete genomes from the organism of interest are available, we identified several target proteins in endo- and ectoparasites using bioinformatic sequence comparison methods. These target proteins formed the basis for homology modeling approaches leading to protein structures which were then used in subsequent docking experiments to identify potential lead structures being able to inhibit the proteins (Figure 4.2) [28].

ESTs from different parasites were downloaded from the dbEST section of GenBank [29] and assembled using CAP3 software [30] in order to minimize redundancy in the datasets and to build reliable and longer consensus sequences. Subsequently, the comparative genomics Genlight software [31] was used to identify

Figure 4.2 Combined *in silico* workflow. After assembly of EST sequences, the resulting contigs and singletons are used to identify potential target proteins. BLASTP searches against the protein structure database PDB revealed crystal structures of homologous proteins, which can serve as templates (red) for protein homology modeling experiments. These protein model structures (blue) can in turn be used for virtual high-throughput experiments like docking. For molecular docking experiments the two-dimensional ligand structures have to be converted into their three-dimensional form.

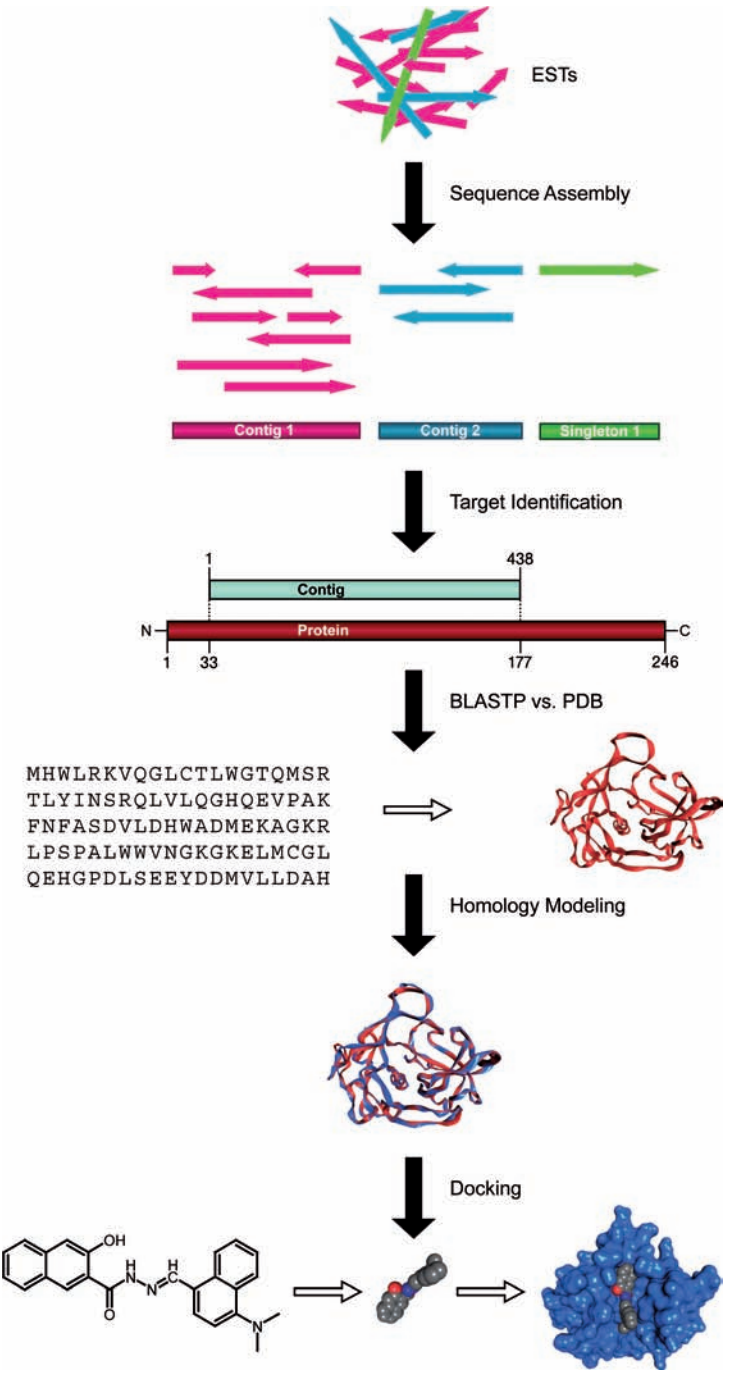
ESTs
Sequence Assembly
Contig 1
Contig 2
Singleton 1
Target Identification
1
438
Contig
N
Protein
C
1
33
177
246
BLASTP vs. PDB
MHWLRKVQGLCTLWGTQMSR
TLYINSRQLVLQGHQEVPAK
FNFASDVLDHWADMEKAGKR
LPSPALWWVNGKGKELMCGL
QEHGPDLSEEYDDMVLLDAH
Homology Modeling
Docking
OH
H
N
N
H
C
O
N

Table 4.1 Number of EST sequences.

Organism	Sequences before assembly	Sequences after assembly	Orthologous proteins
A. suum	39 242	7622	
B. malayi	23 887	8297	271
H. contortus	20 439	4112	
R. appendiculatus	19 046	7349	945
B. microplus	20 560	8074	

target proteins in nematodes (*Ascaris suum*, *Brugia malayi*, *Haemonchus contortus*) and ticks (*Rhipicephalus appendiculatus*, *Boophilus microplus*). At the beginning of the target identification step, orthologous sequences within the respective endo- and ectoparasites were determined. This was accomplished by conducting bidirectional BLAST searches [32] and selecting those sequences which found each other as best hits. The determination of orthologous sequences was necessary to assure that target proteins could be identified which occur in different parasite species, thereby facilitating the development of broad-spectrum drugs. Applying a 30% coverage filter to the bidirectional best hits we found 271 orthologous proteins within the analyzed nematode sequences and 946 orthologs within the tick sequences, respectively (Table 4.1).

To verify whether the orthologous proteins have important functions in the parasites, they were compared with phenotype databases using BLAST searches (e-value cutoff 1 e-35). These sequence datasets, which comprised sequences from *C. elegans* and *D. melanogaster* with phenotypes like *uncoordinated*, *paralyzed*, *lethal*, *neurophysiology defective*, and so on, were collected from WormBase [33] and FlyBase [34]. Proteins with high similarity to sequences in the phenotype databases were selected due to their potential to act as target proteins with essential functions in the target organisms and compared to a human proteome dataset in order to judge their toxicity potential. This comparison made it possible to classify the sequences into two categories (high *vs.* low similarity to vertebrate proteins) and to prioritize the target proteins. As mentioned above, a low degree of conservation to vertebrates is favored in order to minimize toxicity problems for the host organism – so these targets are of higher priority. However, some parasite target proteins are known which differ only in a few amino acids from their host equivalent, still allowing the design of specific drugs, for example, β-tubulin, the target of benzimidazoles [35]. Therefore, we did not want to omit such proteins and applied this classification system based on BLAST alignments and a bit-score threshold filter to distinguish between different levels of conservation. Once identified, the potential target proteins were subjected to in-depth bioinformatic analysis including multiple sequence alignments, database searches in PROSITE [36] and InterPro [37], determination of protein domains and protein localization, and so on. To examine whether suitable 3D structures were available for the respective target proteins, they were compared to the PDB 3D structure database [38]. Such structures can be used for homology modeling or docking

Table 4.2 Number of potential target proteins in endo- and ectoparasites.

Similarity to mammalian proteins	Numbers of queries with hits in phenotype database	
	All queries	**Queries with hits in 3D structure database**
High	Ecto: 65 Endo: 26	Ecto: 41 Endo: 19
Low	Ecto: 15 Endo: 28	Ecto: 4 Endo: 2

approaches [39, 40]. For details on materials and methods, please refer to Krasky *et al*. [28]. Employing this workflow we could identify several potential target proteins in endo- and ectoparasites (Table 4.2), for example, the enzyme S-adenosyl-L-methionine synthetase (SAM synthetase, EC 2.5.1.6). This enzyme was identified in the ectoparasite target group, showed high similarity to vertebrate proteins and was selected to illustrate homology modeling and docking techniques.

SAM Synthetase as a Potential Target for Rational Drug Design

SAM synthetase catalyzes the formation of S-adenosyl-L-methionine from ATP and L-methionine [41]. S-Adenosyl-L-methionine is an essential methyl group donor in biological systems and is involved in the methylation of noradrenalin, DNA, and ribosomal RNA, as well as in the synthesis of phosphatidylcholine [42, 43]. A key prerequisite for potential target proteins is the crucial function for the parasite. For SAM synthetase, lethal phenotypes are described for *D. melanogaster*, suggesting a very important function of this enzyme also for other arthropods including parasites (e.g., ticks).

An additional challenge regarding the quality of a target protein is the degree of conservation. Bioinformatic sequence analysis revealed that this ubiquitous protein is highly conserved. Figure 4.3 shows a multiple alignment of SAM synthetase sequences from the different species *R. norvegicus*, *D. melanogaster*, *B. microplus*, and *R. appendiculatus*. Comparison of the rat SAM synthetase with the respective enzymes of the model organism and the ticks revealed that the vertebrate protein differs in 123 positions from the arthropod sequences and therefore, approximately 69% of the residues are conserved. Nevertheless, we could identify some remarkable differences between the vertebrate and the arthropod sequences which could be exploited for the rational design of specific drugs, for example, the replacement of Cys61 (numbering of the amino acids corresponds to the rat sequence) by Ala71 in *D. melanogaster* and the substitutions in the region from Asp117 to Glu128 forming an extended loop which might be involved in the catalytic activity of the enzyme [44]. Moreover, some amino acids neighboring the active site residues are not conserved,

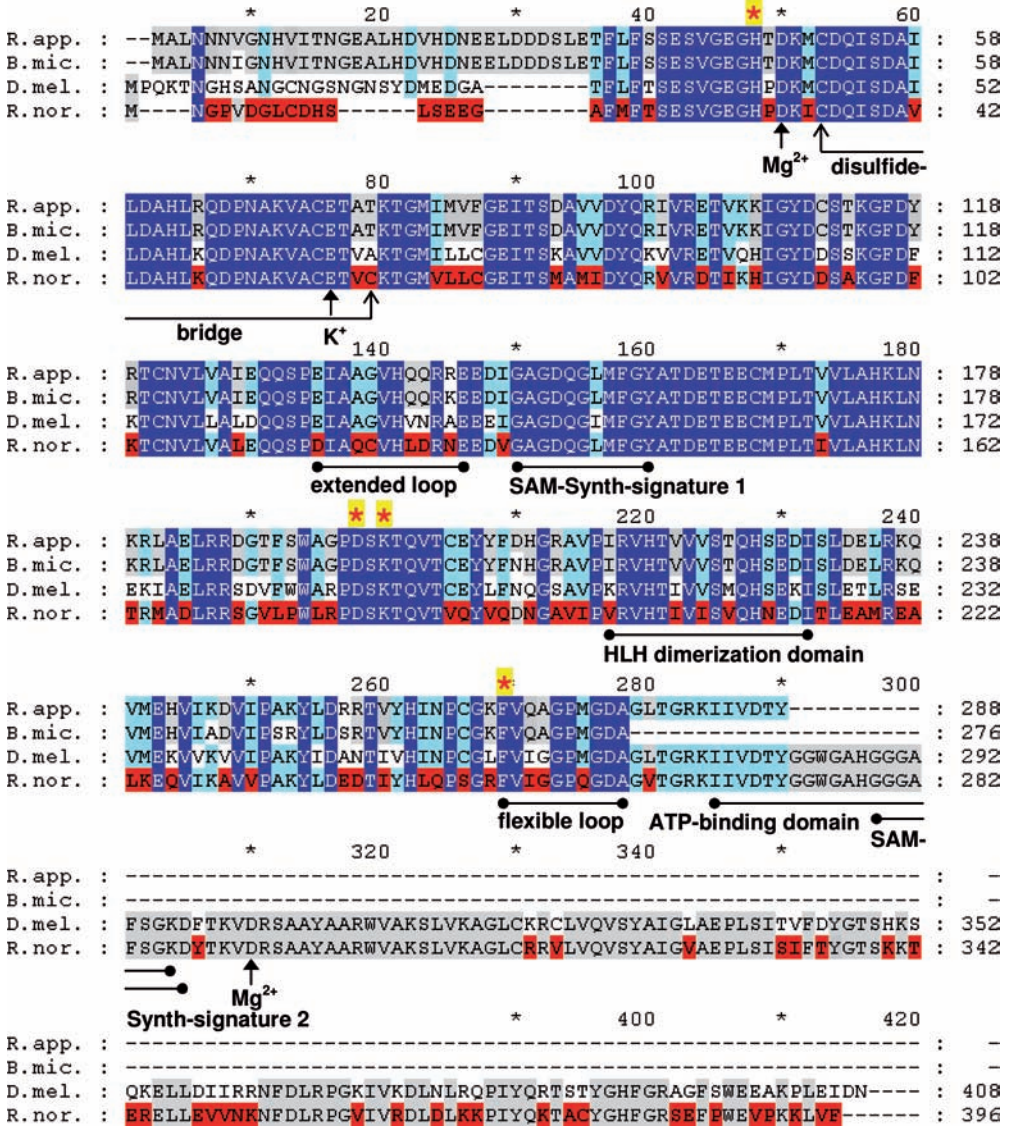

Figure 4.3 Multiple sequence alignment between translated tick EST sequences and SAM synthetase sequences from *D. melanogaster* (Swiss-Prot entry P40320) and *R. norvegicus* (Swiss-Prot entry P13444). Blue-shaded amino acids represent the highest level of conservation while light blue- and gray-shaded residues correspond to a medium and low level of conservation. Red-shaded amino acids indicate positions at which the SAM synthetase sequence from *R. norvegicus* differs from arthropod sequences. Amino acids which belong to the active site of the enzyme (His30, Asp180, Lys182, Phe251) are marked by yellow-shaded red asterisks (the residue numbering corresponds to the sequence of *R. norvegicus*). Furthermore, amino acids binding Mg^{2+} and K^{+} ions which are needed for enzymatic activity are marked by arrows. A disulfide bridge occurs between Cys35 and Cys61 and a flexible loop region can be found

for example, Leu177, Val187, Gln188 or Arg250, which may also be used as starting points for the design of specific antiparasitic drugs.

To identify lead structures via *in silico* docking approaches, a 3D structure of the target protein is an essential requirement. For SAM synthetase, only the crystal structures from the bacterial, the yeast, the human, and the rat enzyme are available, but no structures from invertebrate SAM synthetases. At this point, homology modeling comes into play. This technique is able to deduce the potential 3D structure of a protein using another protein structure as a template [45]. On the basis of the rat enzyme structure (PDB entry 1qm4), which showed the highest conservation to the fruitfly sequence (74% sequence identity), we were able to generate a homology model of the SAM synthetase from *D. melanogaster*. The fact that both proteins show a high degree of similarity on sequence level allows for the generation of very precise homology models [46]. An overlay of the homology model and the template structure shows quite a high degree of similarity at the structural level (Figure 4.4a). This applies to the protein backbones as well as to the side chains of the active site residues.

As soon as a protein structure is available, it can be used for molecular docking experiments. In order to validate whether the present SAM synthetase model structure is suitable for such approaches, we performed docking experiments with L-2-amino-4-methoxy-*cis*-but-3-enoic acid (L-*cis*-AMB), which is a cocrystallized inhibitor of the rat enzyme. A Mg^{2+} ion, which is part of the active site, was placed into the model structure considering its position in the template structure. After the docking experiments the resulting poses of the inhibitor in the protein structure were scored. A visual inspection of the poses revealed that most of them resemble the position of the inhibitor in the original protein structure (RMSD value of the best scored pose: 0.38 nm/3.8 Å). Although this seems to be a rather high RMSD value due to the metal ion, which is difficult to handle by current docking applications, it is still acceptable, because it is very close to the best redocking RMSD value of the originally cocrystallized compound. Moreover, chemical properties play of course a key role in the interactions between inhibitor and protein. Hence, a molecular lipophilicity potential was mapped onto the solvent-accessible surfaces of the inhibitor and the protein (Figure 4.4b) [47, 48] showing that hydrophilic regions of both reaction partners are very close to each other and likewise the same applies to lipophilic regions, so that optimal interactions between the inhibitor L-*cis*-AMB and SAM synthetase from *D. melanogaster* can occur. A more detailed analysis (Figure 4.4c) elucidated that the active site residues (His30, Asp180, Lys182, Phe251) are located in immediate vicinity to the inhibitor molecule. Furthermore, the Mg^{2+} ion is situated near the carboxylate group of the inhibitor molecule, allowing the

between Phe251 and Ala260. In addition, the region from Val199 to Ile214 forms a HLH (helix-loop-helix) dimerization domain. Two characteristic regions, which are conserved in all SAM synthetase sequences, the SAM synthetase-signatures 1 and 2, reach from Gly132 to Tyr142 and from Gly279 to Asp287. An ATP-binding domain is formed by the region Ile267 to Lys286. The C-terminal part of the tick sequences is shorter due to incomplete coverage by the EST sequencing method.

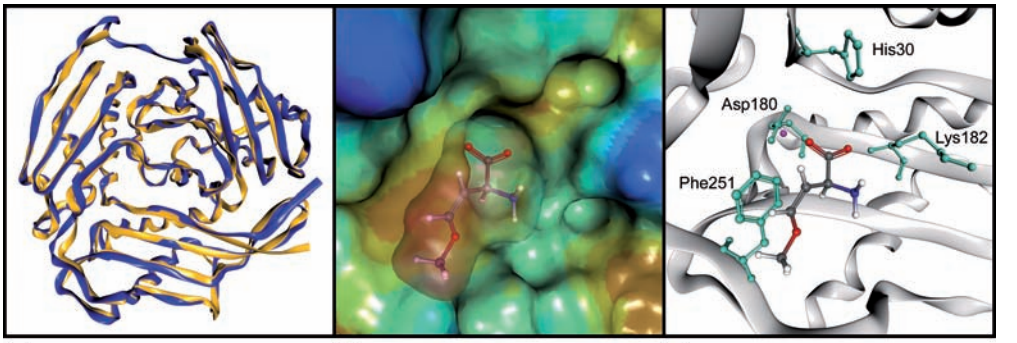

Figure 4.4 3D structures of SAM synthetase. (a) Homology model of SAM synthetase from *D. melanogaster* (blue) and template structure from *R. norvegicus* (yellow) in ribbon-type rendering. A superimposition of the homology model and the template structure (both shown as monomers) reveals almost identical folding patterns between the two proteins. *In vivo* this enzyme forms homodimers or homotetramers. (b) Active site pocket with the best ranked docking solution. Lipophilic regions of both reaction partners are shown in brown and hydrophilic regions in blue. Hydrophilic regions of the protein are neighboring corresponding hydrophilic regions of the inhibitor molecule (L-*cis*AMB). The same applies for lipophilic regions. (c) Active site pocket in detail. The amino acids His, Asp, Lys and Phe (cyan) which belong to the active site and the inhibitor molecule (CPK coloring) are shown in ball and stick. A Mg^{2+} ion which is also part of the active site and plays a role in the coordinative binding mode of the inhibitor is shown in magenta.

coordinative binding mode, which is described in literature [44]. These data show that the inhibitor of the rat SAM synthetase (L-*cis*-AMB) should inhibit the enzyme of *D. melanogaster* as well and that the homology model structure of this protein should be applicable for high-throughput molecular docking approaches aiming at the identification of novel lead structures.

Conclusion

The relatively new scientific fields bio- and chemoinformatics offer valuable tools for the handling, storage, and analysis of large amounts of biological and chemical data. The above-mentioned case study shows how a combination of both disciplines can support and improve the whole drug discovery process. Starting from target protein identification and ending with the optimization of lead structures which can finally be developed to drug candidates, *in silico* methods like comparative sequence analysis, homology modeling, and molecular docking play an important role and have emerged to key technologies which are essential to modern drug discovery.

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5
Target Identification and Validation in Antiparasitic Drug Discovery

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Abstract

Over recent decades, the animal health industry has managed to bring only a few novel classes of antiparasitic drugs to market, reflecting the difficulty of discovering safe and effective molecules. Consequently, a small number of companies shifted the screening paradigm from classic *in vivo* antiparasite screens towards target-based high-throughput screening. The human pharmaceutical industry has undertaken this shift before but is now facing criticism because of the less than expected outcome. Owing to specific characteristics of antiparasitic drug research, the animal health industry is likely to develop a promising version of target-based drug discovery. It combines genome information of parasites and their closely related model organisms with comprehensive target validation tools and real-time testing of target-active compounds in disease relevant bioassays.

Introduction

Today, target-based drug discovery is the dominant approach in the human health drug discovery, replacing the traditional physiology-based approach [1]. It has made increased screening capacity possible and the definition of rational drug discovery programs. In recent years however, target-based drug discovery faced criticism concerning its disappointing return of investment and long timelines [2, 3]. The reasons for this are manifold. On the chemistry side, novel lead compounds have often been difficult to find, either because the chemical libraries are unsuitable for the target class or the screening target turns out not to be druggable. From a risk management point of view, lead molecules selected from target screens can only be tested in disease specific animal models quite late in the drug discovery process, making the failure of the compound an expensive experience. Furthermore, the ability to prevent off-target effects has been poorer than expected despite this being

one of the perceived advantages of the target-based approach. A general scepticism is also nurtured by the fact that many companies work on the same targets. If those targets prove to be fruitless (and many have been) a sense of industry-wide failure prevails [1]. Possibly, the most significant reason for failure is poor validation of screening targets, as this renders all efforts fruitless from the start. The lack of suitable disease models, incomplete understanding of the complex processes causing a disease state, and the misinterpretation of results from expression profiling and knockout experiments are the main handicaps to the validity of screening targets. As a result, 95% of all drugs tested in preclinical trials do not translate into clinical efficacy in human patients [4, 5].

For many years, the target-based approach was not employed in the animal health industry, mainly because effective drug candidates could be recruited from crop protection companies or by repositioning human drugs. These drugs were not identified and optimized starting with the respective molecular target but by *in vivo* screens: Chemical compounds were applied to relevant parasites and resulting outcomes (e.g., paralysis, lethality) were monitored [6]. However, the number of chemical classes identified by this classic approach was limited and established drugs do not match all market requirements. In livestock especially, resistance to marketed products emerges and develops an economic impact [7–10], (see also Chapter 2). Thus, there is strong demand for novel antiparasitic drugs. Because of the limited success of *in vivo* screens to identify novel structural classes, some animal health companies have responded by developing target-based drug discovery programs. Owing to specific characteristics of the animal health setting and driven by a systematic learning process, target-based drug discovery in the animal health industry has developed an approach of its own which is likely to live up to its promises.

Here, we focus on the target based approach of animal health antiparasitic drug discovery. We describe specific features that contrast with human health target-based drug discovery and explain why the target-based approach is particularly suitable for antiparasitic drug discovery in animal health.

Why is the Target-Based Approach Particularly Suitable for Antiparasitic Drug Discovery in the Animal Health Industry?

Both in human and animal health drug discovery, the target-based approach is built on the hypothesis that a given target is suitable for screening specific compounds aiming to achieve a specific therapeutic goal (e.g., curing a disease or killing a parasite). To minimize the risk of choosing a target insufficiently connected to the desired therapeutic response, it is necessary to thoroughly validate a target before it is used in a screening campaign. Since even the most scrutinized hypothesis carries uncertainty, the quality of any target has to be confirmed by applying target-active compounds to biological systems. The sooner this translation from on-target to

biological activity occurs, the more affordable a failure becomes. There are features which distinguish antiparasitic animal health from human health drug discovery that are likely to significantly improve the chance of success.

(i) The desired property of an antiparasitic drug is comparatively easy to define: killing or dispelling parasites within a reasonably sized therapeutic window. It is therefore not necessary to identify targets which modify a diseased condition of the organism. This has proved notoriously difficult in human health drug discovery as it requires the modulation of complex, interdependent systems. Although killing a parasitic organism within or on its host is not trivial, it is relatively straight forward to identify molecular targets which are *per se* essential for the fitness and survival of the parasite.

(ii) The parasitic model systems *D. melanogaster* and *C. elegans* are well characterized, easy to manipulate and, above all, sufficiently close to their parasitic counterparts (arthropods, helminths) to draw valid conclusions. Clearly, some aspects of parasite biology can not be reproduced with free living model organisms such as the mechanisms of immune evasion and aspects of feeding and digestion, for example. For many aspects of core biology, however, it is reasonable to believe that these model organisms are valid indicators. This is emphasized by the fact that virtually all marketed products against ectoparasites or nematodes exhibit a lethal or at least a clear behavioral phenotypic effect on *D. melanogaste* or *C. elegans* (our observations, Table 5.1). Furthermore, the mechanism of drug resistance is often conserved on the molecular level between model organisms and parasites [7, 11–13]. In addition marketed products as well as compounds in development mediate similar activity on their particular molecular target in model organisms as well as on the orthologous targets of parasitic origin (Intervet Innovation, unpublished data).

(iii) In comparison to human health drug discovery, the translation from on-target activity of compounds to biological or clinical activity occurs faster. The target-based approach delivers novel compounds with on target activity rapidly by high-throughput screening (HTS). This advantage can turn into a shortcoming, when

Table 5.1 Established antiparasitic market products affect model organisms.

Market Product	Mode of Action	*D. melanogaster*	*C. elegans*
Imidacloprid	Nicotinic receptor	+	n.t.
Amitraz	Octopamine receptor	−	n.t.
Dichlorvos	Acetylcholinesterase	+	+
Deltamethrin	Sodium channel	+	n.t.
Ivermectin	Glutamate-gated chloride channel	+	+
Fipronil	GABA-gated chloride channel	+	n.t.

Experiments performed at Intervet Innovation. Mode of actions summarized by "Insecticide Resistance Action Committee" (www.irac-online.org/): +, significant effects in Intervets contact or feeding assays; n.t., not tested.

the underlying hypothesis can not be tested promptly in the clinical situation. In the animal health setting, target-active compounds can be tested directly on parasites both in culture and in animal models sufficiently close to the field situation. In addition to this qualitative advantage, there are also quantitative aspects which facilitate the confirmation of the target validity: comparatively small amounts of compounds identified in screening can be tested promptly in simple antiparasitic assays to assess their biological activity. In contrast to this, it is the general experience in human health drug discovery that the simulation of a human disease in rodents, swine, dogs or primates is technically demanding in that it requires disease specific genetic, mechanical or pharmacological manipulations to mimic the clinical situation. In addition, such disease models require larger amounts of compound and are therefore used relatively late in the drug discovery workflow [14]. Consequently in human health drug discovery, the validity of the screened target is assessed later and with higher uncertainty.

Special Challenges for AntiParasitic Drug Discovery in Animal Health

There are however some features of antiparasitic drug discovery which create a more demanding starting point for the animal health target-based approach. Foremost, is the goal to discover drugs against a wide spectrum of target organisms, often occupying different niches. Whereas human health drug discovery can focus on one target organism, the animal health industry seeks to cover species as diverse as flies, fleas, and ticks in the ectoparasitic field, as well as nematodes, trematodes, and cestodes in the endoparasitic field. The situation is complicated by the fact that the spectrum of patients includes fish, companion animals and livestock animals. Whilst the spectrum of patients poses a generic challenge for medicinal chemistry, the wide spectrum of target parasites demands a decision as to whether to select a representative target protein (e.g., an ion channel from *D. melanogaster* as the target for ticks, fleas, and flies) or to use orthologous targets from representative parasitic species to identify overlapping hits. In our experience, screening with parasitic targets increases the chance of identifying bioactive substances with the desired spectrum and allows rational decision making when it comes to lead optimization because parasitic on target activity can be correlated with parasitic *in vivo* activity.

Properties of a Valid AntiParasitic Screening Target

A valid antiparasitic screening target has to fulfill certain criteria (Table 5.2): The target should be required for survival or fitness of the parasite in the relevant

Table 5.2 Criteria ideal antiparasitic screening targets have to fulfill.

Criteria	Critical question
Biological function	Is there any evidence that target is essential for growth or survival?
	Is the target expressed in parasite life cycle suitable for drug intervention?
Assayability	Is HTS screening feasible?
Potential for resistance development	Are biochemical bypass reactions known?
Biochemical properties	Is the target function rate limiting in its pathway?
	Will target turnover allow inhibition over a reasonable time period?
Selectivity	Is the target absent in mammals?
	Are molecular or pharmacological properties distinct from related host protein?
Structure and druggability	Is a crystal structure obtainable, preferably with bound cofactor, inhibitor or agonist?
	Does the target have a small molecule ligand-binding pocket?

developmental stages. These would be the blood-sucking and reproducing stages for fleas and ticks and the larval and adult stages for pest flies. For endoparasites like nematodes the situation is similar in that late larval and adult stages are most important for fast-acting intervention. This criterion is critical for the success of a project as it can not be compensated for later in the drug development process. Equally important is the suitability of a target for HTS screening. Comparable to human health drug discovery, the assay repertoire spans ion channels, GPCRs, and enzymes. In human health drug discovery, only one high-throughput assay per target has to be established whereas the animal health situation requires that the targets of several representative parasitic species be cloned and functionally expressed.

Another criterion is the extent to which a given drug target is prone to the development of drug resistance. This aspect is of particular importance as the development of resistance against current antiparasitics is one of the driving forces of the search for new chemical classes or modes of action. There are some examples in the literature where target site resistance could be induced under controlled conditions [7, 15]. Furthermore, genetic prediction tools for resistance development have been established [16]. However, we believe it will remain difficult to realistically estimate the potential for resistance in novel targets.

Also of importance for the success of a project are whether a target function is rate limiting in its pathway, whether there is redundancy, and whether the target turnover allows inhibition over a reasonable time period. Finally, there are criteria such as selectivity and structural information which can improve the success probability but are too rare to apply as a strict rule.

Why Are Novel Targets Needed

The targets with the highest validation status are the classic antiparasitic targets chemically validated by marketed compounds; for example, GABA-gated chloride channel, glutamate-gated chloride channel, nicotinic acetylcholine receptor, or acetylcholine esterase. Why not focus on these well validated, classic targets to identify novel antiparasitic drugs? These targets have proven to be critical for the survival of the relevant parasite stages. They are druggable and allow for selectivity against the host. This seemingly ideal situation has severe drawbacks. If resistance is arising or already present in the field, a target-based HTS screen using the respective molecular targets involves the risk of identifying compounds to which field populations are also resistant. This drawback can be converted to an advantage by screening against a resistant variant of the molecular target. For example, a point mutation in the GABA-gated chloride channel in insects results in resistance to Dieldrin and reduces the sensitivity to Fipronil [17–19]. Choosing the resistant variant of the GABA-gated chloride channel for HTS screening will result in drugs that overcome the known resistance. Staying with Fipronil as an example again, potent marketed products are often not specific for only one molecular target. Fipronil antagonizes GABA-gated chloride channels but at the same time agonizes glutamate-gated chloride channels [20]. Therefore, the correlation of on-target activity with *in vivo* activity seems to be difficult and bears the risk of overestimating the importance of one of the involved molecular targets. The number of targets that are chemically validated by marketed products is extremely limited. Taken together, to concentrate on well established antiparasitic targets alone is insufficient to realize the potential of the target-based approach. Thus, it appears that a rewarding albeit demanding challenge for target-based drug discovery is to identify novel drug targets.

Target Identification Techniques

The genome of *D. melanogaster* comprises approx. 14 000 predicted genes and the genome of *C. elegans* about 19 000 genes. The genomes of the related parasitic species are expected to be of similar or even higher complexity. Current knowledge on nearly all genes described in the scientific literature is summarized and assembled in databases at NCBI (www.ncbi.nlm.nih.gov/), FLYBASE (www.flybase.net/), INTERACTIVE FLY (http://www.sdbonline.org/fly/aimain/1aahome.htm), FLYMINE (www.flymine.org/) and WORMBASE (www.wormbase.org/). Therefore, these databases are an ideal starting point for initiating a target validation project. Potential targets can be filtered out of these databases by a combination of search items and the interpretation of phenotype descriptions such as expression in relevant stages and tissues, conditional phenotypes, lethality, or effects on locomotion. Of particular value are annotations describing the interaction of drugs with

individual gene products. A prominent example is the GABA-gated chloride channel in *D. melanogaster*, which was named RDL (resistant to dieldrin) after its antagonist Dieldrin [17, 18]. A potential source for novel targets is the cholinergic system in *C. elegans* where mutations in numerous genes affect the sensitivity to levamisole [21, 22]. In a second step, the existence of orthologous genes in relevant parasites is determined by BLAST analysis. In case the desired information is not available, we identify and clone orthologs by degenerate and RACE-PCR.

Alternatively, the use of elaborate bioinformatics tools can be a starting point for identifying novel targets. We performed genome-wide blast analysis to identify genes which are highly conserved throughout parasites and model organisms but are ideally absent in mammalian species or show low similarity to mammalian orthologs [23], (see Chapter 4). Unfortunately, the outcome of this analysis was poor in that for most resulting potential targets no annotation was available. In other words, the molecular function and the related phenotypes were unknown for most of these candidates. However, future functional characterization might promote these genes to become starting points for target-based screening programs.

Techniques to Validate Antagonist Targets

About 10 years ago crop science companies entered into the target-based approach. At that time industry scientists started to use genetic systems in model organism to identify and validate insecticide targets. This arsenal consists of several knockout collections created by chemical or transposon-mediated mutagenesis [24, 25]. The combined mutant collections provided a large although not genome-wide coverage of known genes. Over the years each mutation has been characterized in great detail with respect to phenotype, molecular impact on gene structure and genetic interaction with other mutations (www.flybase.net/, www.wormbase.org/). However, it turned out that the tools are not sufficient to match all target validation requirements: Knockout mutants provide information about the earliest critical developmental stage when the lack of gene function causes a detectable phenotype. Where this phenotype causes embryonic or larval lethality, it is very likely that the gene has developmental functions or plays a role in the fitness of early larvae [25]. Where individual animals reach adult stages (escapers), reduced fitness is often caused by early misdevelopment rather than the acute lack of the protein under investigation. Therefore, it is usually not possible to predict from knockout phenotypes whether a protein is essential in late larval and adult stages, the very stages that are critical for parasitic control. Even if knockout mutations could be induced in adult animals there remains the problem that a complete knockout does not sufficiently match the reality of drug action. An antagonistic drug hardly ever blocks the activity of its target protein in all tissues to zero; rather it lowers its activity.

A comparable reduction is accomplished by genetic knock-down approaches. A prominent technique is RNA interference (RNAi), which combines accessibility of

all potential genes present in a genome with drug-like reduction of target activity. Initially discovered in *C. elegans* [26], RNAi was soon established in *D. melanogaster* [27] and to some extent also in multicellular parasites [28–30]. In the nematodes *C. elegans* this technique is theoretically able to analyze an antagonist target at any developmental stage as double-stranded RNA (dsRNA) can be supplied via injection or feeding of dsRNA producing bacteria [31, 32]. In insects, dsRNA has to be provided by transgenes since dsRNA is not actively transported across cell membranes and is therefore insufficiently distributed on feeding or injection [33].

We tested transgenic RNAi in *D. melanogaster* to assess its suitability as a genetic validation tool. We chose 10 diverse genes with a lethal phenotype induced by transposon insertion and established transgenic fly lines expressing target-gene-specific dsRNA under the control of upstream activating sequences (UAS, derived from yeast and not present in *Drosophila melanogaster*) [34]. The production of dsRNA is silent in these flies but can be turned on by crossing with driver fly lines expressing a specific transcription factor (GAL4, derived from yeast and not present in *Drosophila melanogaster*; Figure 5.1) [34]. When we used ubiquitous GAL4-driver fly lines (actin-GAL4 and tubulin-GAL4) we found that in eight out of 10 genes the expected embryonic/larval lethality was reproduced by transgenic RNAi, indicating that this system is as powerful as genetic knockouts. Transgenic RNAi has the additional advantage that the gene dosage is not reduced to zero and is therefore close to a drug action and all potential target genes can now be analyzed by this technology. Over recent years laborious genome-wide RNAi knockdown approaches also based on the UAS/GAL4 system have been established [35] (http://www.shigen.nig.ac.jp/fly/nigfly/index.jsp and www.vdrc.at/). These collections could be a valuable and time-saving source to validate any potential target gene. However, the transgenic RNAi system induces lethality during the first essential expression of the particular target and therefore obscures the analysis in the relevant stages. Therefore, we had to avoid the knockdown in embryonic stages and induced RNAi only in late larvae or adult animals. This was achieved by controlling the expression of dsRNA via the GENESWITCH system where a modified GAL4 activator is induced by feeding the hormone RU486 [36] or via the combination of the original UAS/GAL4 system with a temperature-sensitive repressor (GAL80ts, derived from a mutant yeast strain and not present in *Drosophila melanogaster*) [37] (Figure 5.1). In both systems we could show that embryonic and larval phenotypes could be avoided and that temperature shift or hormone feeding induced specific RNAi mediated phenotypes in adults. This elaborate induction system is, as expected, not as penetrant as the non-inducible RNAi systems but constitutes for us the state-of-the-art genetic validation system for potential antagonist targets.

A similar knockdown technique, but independent of RNAi, could be deduced from a method recently published by Zeidler *et al*. [38]. They combined temperature-sensitive intein protein-splicing elements (protein elements capable of self-excision) with a GAL4 activator and a GAL80 repressor. In this system, the target under investigation could be expressed in a null mutant background under the control of a suitable GAL4 driver. Under permissive temperature, the GAL80 repressor is activated, causing a knockdown of target gene expression. Furthermore, established

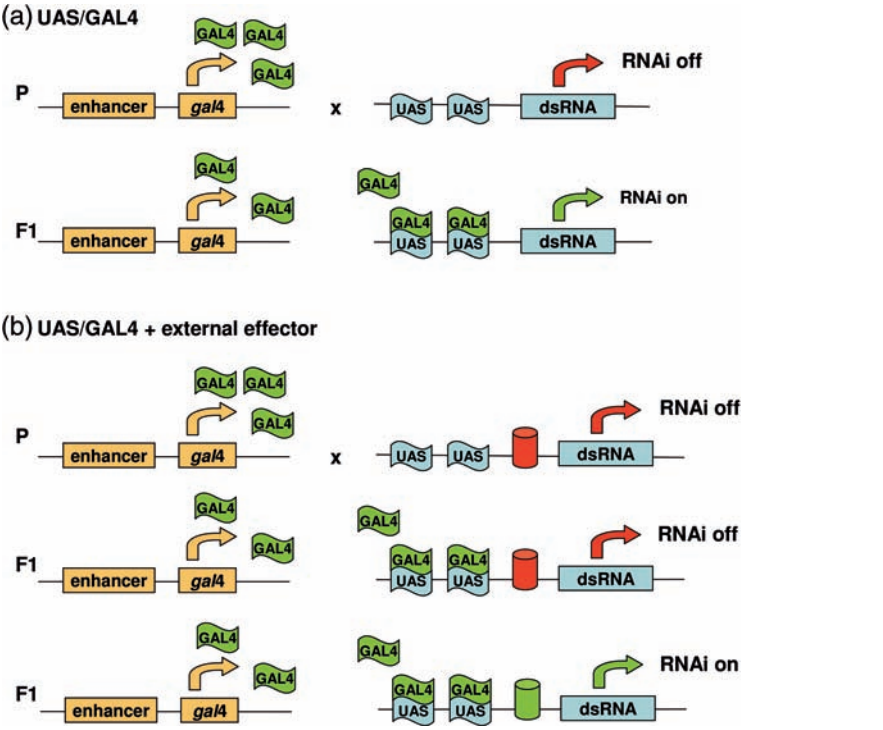

Figure 5.1 Induction of transgenic RNAi in *Drosophila melanogaster*. (a) The UAS/GAL4-system [34] is used to knock down a specific gene in the progeny generation by crossing a driver fly expressing the transcription factor GAL4 with an effector fly line expressing gene specific dsRNA. Thereby, the expression of dsRNA is regulated temporally and spatially by the enhancer that regulates expression of GAL4. (b) The UAS/GAL4 system can be modified by the use of an external effector like feeding a hormone [36] or a temperature [37] shift. This external effector is symbolically depicted by a barrel. The expression of dsRNA is regulated spatially by the enhancer that regulates expression of GAL4 and temporally by the external effector. Using a ubiquitous driver line (e.g., tubulin-gal4, actin-gal4) in combination with an external effector allows turning on the RNAi mechanism for a particular gene ubiquitously at any time of development.

techniques like Cre-*lox P* and Flp-*frt* or the Tet-On and Tet-Off-system [39–41] could be used to develop inducible validation tools.

Some drawbacks common to all knockdown approaches might lead to false-negative results and have to be taken into consideration. For some genes RNAi appears to mediate weak knockdowns resulting in weak phenotypes. In addition, in heterodimeric receptor complexes, the knockdown of a single subunit could be compensated by homodimeric or alternative combinations. Also, slow target protein turnover rates could prevent the detection of a phenotype within a reasonable observation period. Alternative approaches might prevent these potential targets from being rejected. Unfortunately, these techniques are labor intensive and/or not applicable for all potential target genes. Most of the problems stated above could be

overcome when a conditional loss of function is directly induced in the target protein. Although temperature-sensitive loss of function mutations are known in model organisms, they do not play an important role in target validation projects as they rarely occur in interesting protein classes without affecting protein function at permissive temperature. Another technique which might be suitable for validating stable target proteins is the degron system, which is based on the mammalian n-end rule protein degradation [42]. To our knowledge, this technique has not been tested in *D. melnogaster* or *C. elegans* yet.

Techniques Which Mimic Agonistic Actions

The possibilities for identifying and validating agonist targets are dramatically different to the situation for antagonist targets. In contrast to antagonistic drug action, mimicked by loss-of-function genetics, the action of agonistic drugs can only be simulated by a more difficult gain-of-function approach. Comparable to the numerous transposon mediated knockout screens [24, 25] random gain-of-function screens using enhancing transposable P elements (EP elements) have been performed [43]. When inserted in the 5′ region of a gene, EP elements are able to mediate over expression via the UAS/GAL4 system (compare above). The weak spot of the EP tool are the GAL4 drivers. Here, the over expression is regulated by the expression pattern mediated by the GAL4 driver line, which is mostly not restricted to the natural tissue, where the target gene is endogenously expressed. As a consequence, the resulting phenotype could be caused by ectopic expression rather than by over expression. Thus, the expression pattern mediated by the GAL4 driver line must be close to the endogenous expression of the target or ideally identical. The GAL4 enhancer trap insertion database (GETDB) of about 7000 lines might serve as source for the identification of a suitable driver line [44]. The construction of the desired driver line by cloning the appropriate enhancer region might be an alternative, albeit time-consuming and demanding. Analogous to the inducible loss-of-function techniques, the overexpression needs to be postponed to larval and adult stages in order to prevent early lethality. The work by Zeidler *et al.* [38] mentioned above appears to provide a solution. They constructed a temperature-inducible GAL4 transcription activation which, when expressed in the tissue of interest, could boost target expression under permissive temperature. However, even if these problems are solved, another difficulty could prevent this approach from being adequate. In cases where the amount of target protein is not rate limiting the mere over expression of a target candidate will not produce any effect. In this case the only solution is the over expression of constitutively active variants of the target proteins [45, 46]. Currently, the knowledge to construct such active forms is not available for most proteins.

A more direct method for identifying agonist targets is the use of validation compounds. Such compounds must fulfill two requirements: they need to be selective and they should be drug-like in order to reach their target, either upon feeding,

contact, or injection. In a first step, the validation compound needs to be identified. Sources can be nature, natural toxins [47], or small chemical libraries focused on attractive protein classes. In a second step the compound is applied to the wild type model organism to test whether it causes the desired phenotype (e.g., paralysis, death, locomotion effects). In order to ensure that the validation compound really mediates its action on the expected target, the substance is tested for agonizing activity on its target *in vitro* and if possible on animals where the target is knocked down. A loss or reduction of drug response in the mutant animal makes it likely that the knockeddown gene is the drug target [7, 48]. A target validated along these lines is a highly valuable target as most of the critical questions listed in Table 5.2 can be answered.

Mode of Action Studies As A Show Case for the Target-Based Approach

An alternative starting point for identifying and validating drug targets are compounds with strong antiparasitic activity but yet unknown mode of action. We initially test such compounds in a compendium of assays representing most known and several assumed antiparasitic targets. In case these tests do not reveal the molecular target or additional targets are suspected, genetic mode of action studies can be carried out, using *D. melanogaster* or *C. elegans* [49]. Typically, a genetic screen using chemical mutagens or transposon mutagenesis is performed in order to induce mutations altering the sensitivity to the compound. Organisms, resistant or hypersensitive to the drug are used to identify and clone the molecular target(s). The number of putative targets emerging from such studies is relatively small (typically 3–10 genes/proteins) – a tractable number for further deconvolution. The targets hit by the compound directly are proven to be druggable and critical in the relevant life stages of parasites.

In recent years, additional techniques based on the physical interaction between compound and proteins have been developed [50]. These technologies require the immobilization or modification of the drug to allow the selection of interacting proteins. Judging from the examples published, the methods will be most successful when the target protein is not membrane-bound like many enzymes.

The most suitable method must be chosen case by case, informed mainly by assumptions about the possible target, the penetrance of the phenotype in model organisms, or the properties of the small molecule. For the animal health industry, compounds with the desired activity on parasites for which the mode of action is unknown are rare. When a company discovers or licenses such a compound, it must decide whether to elucidate the mode of action or start a chemical optimization project without the benefits of knowing the molecular targets. The downside of mode of action studies is clearly the investment of money and time. Depending on the

model organism or the type of affinity based method, an investment of €1–3 million over 1–2 years has to be expected. In addition, the identified target protein might turn out to be not assayable. However the opportunities arising from a known mode of action are manifold:

(i) Drug design is more rational when both biological and on-target activities can be correlated with structural features of a compound.

(ii) The identification of additional compound classes (class hopping) for high-throughput target screening is easier when the mode of action is known. Class hopping is particularly important when the original compound is heavily protected by patents and not accessible by licensing. Further, additional compound classes can help to exploit the full potential of a target by providing alternative physicochemical properties.

(iii) Target-based assays require significantly smaller amounts of compound than bio-screens, thus allowing faster and more diverse chemical modifications.

(iv) Aspects that are related to target-specific toxicity and side-effects can be addressed early by counter-screens, thereby potentially reducing later-stage "attrition" more efficiently.

Conclusion

Despite the impressive progress in the improvement of validation tools, there is no magic bullet for the validation of antiparasitic drug targets. We recommend applying a combination of techniques to test the hypothesis (validate the target) from different starting points. Even if such tests have qualified a target for screening there remains some uncertainty about whether the screen is suitable for identifying selective and efficient compounds. In contrast to human health drug development, the animal health situation allows the resolution of this uncertainty by testing target-active compounds in biological assays, which are well standardized, fast, and highly relevant to the clinical situation. The combination of a relatively simple therapeutic goal (e.g., to kill parasites), the availability of disease relevant bioscreens, and the high validation status of antiparasitic targets make it likely that the target-based approach will generate success stories over the coming years.

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6
Selective Drug Targets in Parasites

Peter Köhler and Richard J. Marhöfer*

Abstract

A strategy to develop new, more selectively acting anti-infective agents is on the basis of identifying key molecules within the pathogen that are non-existent in the host. The detailed knowledge of such targets coupled with combinatorial chemistry, virtual screening or other techniques may then permit the development of new lead compounds. The focus of this chapter is to discuss some examples of novel parasite proteins that appear to have great potential for chemotherapeutic intervention or have already proven as suitable drug targets.

Introduction

Estimates of global parasitism in humans show that protozoan and worm parasites affect several billion people. Parasites cause also serious diseases in domestic animals, resulting in enormous economic losses. Although some of the more recently developed antiparasitic drugs show outstanding chemotherapeutic properties, there remain major deficiencies in the treatment of many parasite-derived infections. Various available drugs have significant side-effects, vary in their efficacy or have lost their value because of the development of resistance. A number of parasitic infections still await the development of clinically effective drugs. For these reasons, efforts to develop new and more selectively acting antiparasitic agents are urgently needed. A strategy to meet this goal is based on identifying molecular structures that could permit a most selective toxic action of a drug against the parasite without harming the host. Such rational approaches to drug design against specific parasite targets are now facilitated through the enormous knowledge of parasites that has accumulated in recent years about their biochemistry, cell biology, and by genome analyses [1–3]. This vast amount of information has uncovered a large number of metabolic pathways and macromolecular structures that distinguish the infective organisms from their respective hosts.

Most of these systems have homologs in the vertebrate host but differ in their structure, functional properties, and relative metabolic importance. Others are not found in higher animals or do not exist in any other forms of life. Many of the latter systems are suggested to have been acquired by gene transfer from bacterial or archaeal donors or have an ancient origin in the eukaryotes. The development of drugs directed against molecules that fulfil essential physiological roles in the pathogen and have no counterparts in the host is of primary importance because of advantages of greater safety and selectivity. The focus of this chapter is to discuss some of these unusual molecular components found among various parasites, all of which are considered as attractive drug targets for structure-based drug design. Brief mention is also made of the most effective inhibitors of these target areas known to date.

Anaerobic Protozoa

Anaerobic protozoan parasites, including *Entamoeba histolytica*, *Giardia lamblia* (syn. *G. intestinalis*), *Trichomonas vaginalis*, and *Tritrichomonas foetus*, share various biological and biochemical features, some of which are highly unusual and are not found in higher animals [1–3]. These organisms differ from most other eukaryotes in that they lack morphologically recognizable mitochondria ("amitochondriates") and consequently possess an entirely fermentative type of energy metabolism. Carbohydrates serve as primary substrates that are degraded via an extended glycolytic pathway with acetate, ethanol, and CO_2 as predominant end-products (Figure 6.1). In this route, a number of enzymes are unusual, including two pyrophosphate-dependent kinases and a ferredoxin-linked enzyme. Another peculiar metabolic aspect of anaerobic protozoa relates to the sulfur-containing amino acid metabolism, where some enzymes are found that are non-existent in higher animals. These include the methionine γ-lyase required for the degradation of methionine and related amino acids and two enzymes involved in the cysteine biosynthetic pathway, serine *O*-acetyltransferase and cysteine synthase.

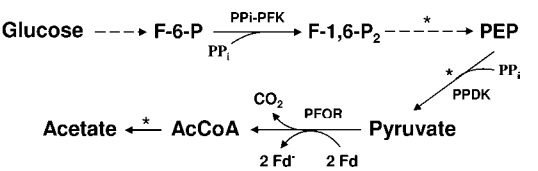

Figure 6.1 Simplified scheme of the core energy metabolism in amitochondriate protozoa. In *Giardia* and *Entamoeba*, the entire pathway occurs in the cytosol, whereas in trichomonads the final steps involved in pyruvate oxidation to acetate occurs within the hydrogenosome and is coupled to hydrogen production (not shown). Broken arrows symbolize multi-step enzymatic reactions. Asterisks indicate sites of ATP formation. AcCoA, acetyl coenzyme A; Fd, ferredoxin; F-6-P, fructose-6-phosphate; F-1,6-P_2, fructose-1,6-bisphosphate; PEP, phosphoenolpyruvate; PPi, pyrophosphate; PPi-PFK, PPi-dependent phosphofructokinase; PFOR, pyruvate ferredoxin oxidoreductase; PPDK, pyruvate phosphate dikinase.

PPi-Dependent Phosphofructokinase (Energy Metabolism)

A notable feature of glycolysis in amitochondriate protozoa is its dependency on pyrophosphate (PPi) as a phosphate donor rather than on adenine nucleotides (Figure 6.1). In upper glycolysis, conventional ATP-dependent phosphofructokinase is replaced by a PPi-dependent phosphofructokinase (PPi-PFK, EC 2.7.1.90) that utilizes PPi to phosphorylate fructose 6-phosphate to form the corresponding l,6-bisphosphate. This enzyme is also found in bacteria, plants, apicomplexan parasites, and in the free-living amoeba *Naegleria fowleri* [4]. Sequence comparisons and phylogenetic reconstruction revealed a significant divergence among these enzymes. Except the plant enzyme, which is an allosterically regulated heterotetramer, all PPi-PFKs are nonallosteric and most are dimeric. Various PPi-PFKs of amitochondriate protozoans have been sequenced and their substrate-binding sites have been analyzed [4–7]. The *E. histolytica* enzyme was found to be a 60 kDa homodimer structurally similar to the enzyme from *Giardia*, while the homologous enzyme from *T. vaginalis* appears to exist as a tetramer of four identical 50 kDa subunits. The crystal structure of the 60 kDa PPi-PFK from the spirochete *Borrelia burgdorferi* has been solved and the reaction mechanism and structural requirements for PPi utilization catalyzed by this enzyme have been demonstrated [8] (Figure 6.2).

The recombinantly produced bacterial enzyme confirms the importance of an Asp and a Lys motif present only in PPi-utilizing PFKs and identifies the structural requirements responsible for sterically blocking the binding of ATP, the phosphorylating substrate of ATP-dependent PFKs. The availability of crystallographic data of the *B. burgdorferi* PPi-PFK improves the feasibility of designing inhibitors specific for PPi-PFKs. Various previous studies attempted in the absence of crystallographic data to identify inhibitors of PPi-PFK [9, 10]. These referred primarily to a group of PPi analogs, such as carbonyldiphosphonate and bisphosphonates that were found to competively inhibit the enzyme with respect to the phosphorylating substrate without affecting the mammalian type ATP-dependent PFK. Some of these compounds showed significant inhibition of *E. histolytica* growth in culture and decreased amoeba-derived liver abscess formation but had no effect on the growth of other parasites not possessing a PPi-PFK, suggesting that the compounds affect the parasite by specifically inhibiting PPi-PFK. The most active compounds were nitrogen-containing bisphosphonates with relatively large aromatic side-chains. The identified PPi analogs have neither been tested clinically for their antiparasitic efficacy nor have attempts since been made to increase the potency of the potential compounds with further chemical modification.

Pyruvate Phosphate Dikinase (Energy Metabolism)

A second PPi-dependent enzyme involved in glycolysis of anaerobic protozoa, pyruvate phosphate dikinase (PPDK, ATP:pyruvate, orthophosphate phosphotransferase, EC 2.7.9.1), is located in lower glycolysis, where it replaces the conventional ATP-dependent pyruvate kinase to generate pyruvate and ATP (Figure 6.1). PPDK

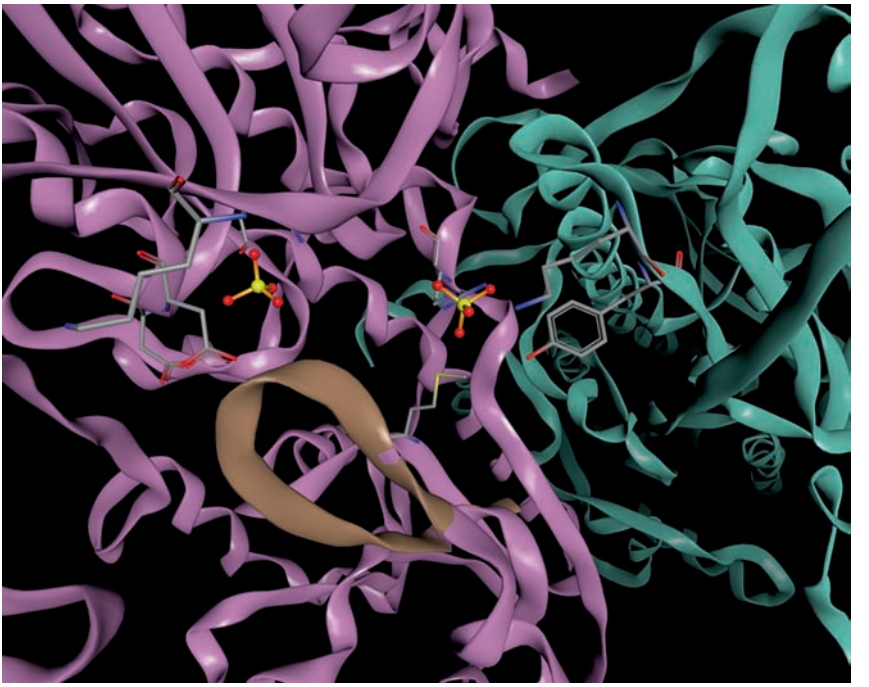

Figure 6.2 Active site of PPi-PFK from *B. burgdorferi*. The active site is located at the interface of the two protein chains (chain A purple, chain B green). The active site residues are depicted in stick representation, sulfate anions resembling the substrate position are depicted in ball and stick representation. The hairpin-loop in front of the active site [8] is highlighted brown. (PDB-Code 1KZH).

catalyzes the reversible conversion of ATP, Pi, and pyruvate into AMP, PPi, and phosphoenolpyruvate (PEP), respectively, by three partial reactions that involve pyrophospho- and phosphoenzyme intermediates. The presence of this enzyme has been demonstrated in bacteria and various amitochondriate protozoa, including *Giardia*, *E. histolytica*, the oxymonad *Streblomastix strix* and, according to recent genetic evidence, also in *T. vaginalis* [11, 12]. PPDK has also been found in the glycosomal compartment of trypanosomes, where it is believed to react in the direction of PEP formation to scavenge PPi [13]. The reverse reaction of the enzyme is also utilized in some bacteria and in chloroplasts of C4 plants to provide PEP for gluconeogenesis. Recombinant PPDK from *Giardia* was purified and its biochemical and solution structure properties characterized [11]. The analysis of the 197 kDa homodimeric enzyme has indicated that the pyruvate-forming reaction is much more efficient than the reverse, PEP-forming reaction. The crystal structures of PPDKs from *Clostridium symbiosum*, *Trypanosoma brucei*, and maize have been solved and the substrate binding sites and mechanism of catalysis have been extensively investigated (Figure 6.3) [13, 14].

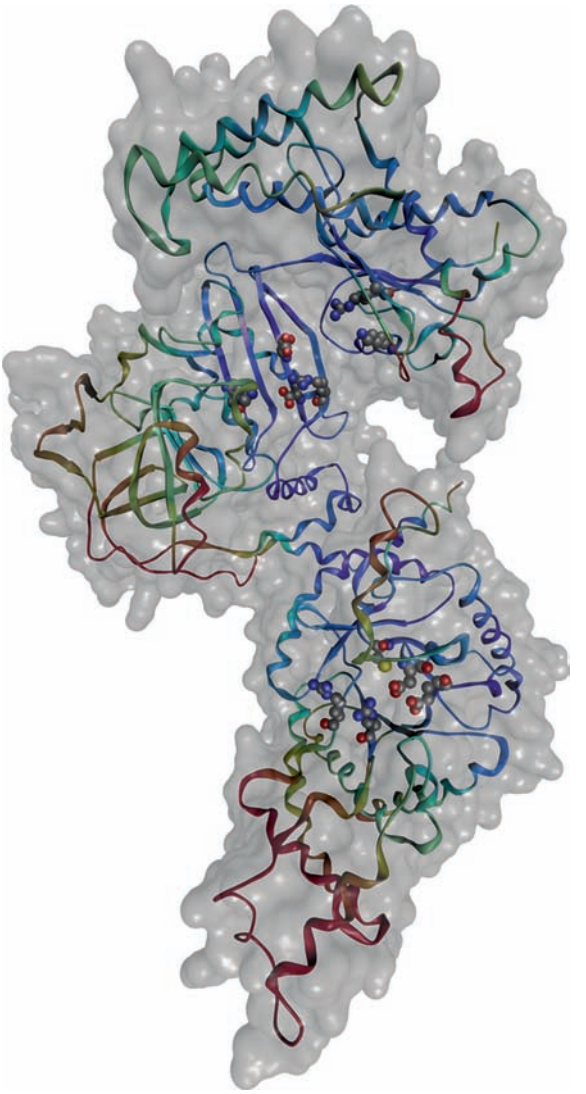

Figure 6.3 The glycosomal pyruvate phosphate dikinase from *T. brucei*. Cartoon representation and solvent accessible surface of the protein chain. Secondary structure elements – α-helices and β-sheets – are shown as helical coils and arrows, respectively. The cartoon is colored according to average temperature factors (blue – low, red – high). Ligand binding and catalytic residues are shown in CPK representation [13]. (PDB-Code 1H6Z).

The sequence similarities observed between the homodimeric PPDKs from different sources suggest that the enzymes are related evolutionarily and have a conserved three-dimensional structure containing an N-terminal nucleotide binding site and a C-terminal pyruvate/PEP binding site. A third region, called the central

domain, contains the acceptor/donor amino acid residues for the phosphate group. Biochemical, mutational, and structural studies suggest that the catalytic activity involves the swivel-motion of a central domain to transfer a phosphate group between two remote active sites. Because of the absence of PPDK in higher animals, this enzyme may, like PPi-PFK, represent a suitable target for antiparasitic drug development. Giardial PPDK was found to be inhibited by imidophosphate, while bisphosphonates were only marginally inhibitory [11]. Recently, the three-dimensional structure of the *E. histolytica* PPDK was modelled and used to carry out virtual screening of compounds taken from small molecule libraries [15]. The interaction studies provided a number of promising ligands for future experimental screening for novel PPDK inhibitors.

Pyruvate Ferredoxin Oxidoreductase (Energy Metabolism)

The recognition of pyruvate:ferredoxin oxidoreductases (PFORs) as sites for inhibition of anaerobic pathogens has resulted in an increased interest in their structure and function. These enzymes are members of the 2-keto acid oxidoreductase family that play a central role in fermentative metabolism of archaea, most anaerobic bacteria and amitochondriate protozoa (Figure 6.1). In these organisms, the key intermediate metabolite, pyruvate, undergoes oxidative decarboxylation by PFOR, a reversible reaction that is coupled to the reduction of a low-potential electron carrier, such as ferredoxins, and the subsequent generation of acetyl-CoA and CO_2. In most aerobic organisms, the oxidative decarboxylation of pyruvate to acetyl-CoA is carried out by the multi-enzyme complex pyruvate dehydrogenase with NAD^+ as electron acceptor. The structure and catalytic mechanism of PFOR and pyruvate dehydrogenase are fundamentally different, the only feature common to both being the requirement of thiamine pyrophosphate as a cofactor for the decarboxylation reaction. From the three phylogenetically related types of PFORs found in different species, anaerobic protozoa contain an enzyme with two identical subunits similar to that found in some anaerobic bacterial sources [16]. In addition to the thiamine pyrophosphate cofactor, PFORs possess suitably arranged 4Fe-4S clusters to provide the electron transfer within the enzyme and to the oxidized ferredoxin. The PFORs and their corresponding genes have been analyzed from *G. lamblia*, *E. histolytica*, and *T. vaginalis* [17, 18]. The homodimeric enzymes were found to be homologous to eubacterial PFORs, membrane-associated and different in the positional identities among the three species. More detailed insights into the structure and catalytic mechanism are available only for prokaryotic PFORs, such as for the sulfate-reducing bacterium *Desulfovibrio africanus* [19].

This enzyme, which is homologous to the protozoan PFORs, was characterized at high resolution by X-ray crystallography and its atomic model complexed with its substrate determined (Figure 6.4). These studies have shown that the catalytic mechanism of PFOR seems to differ remarkably from other enzymes dependent on thiamine pyrophosphate that undergoes a unique conformational change in PFORs. As PFOR fulfils a vital metabolic function for all anaerobic protozoa and has

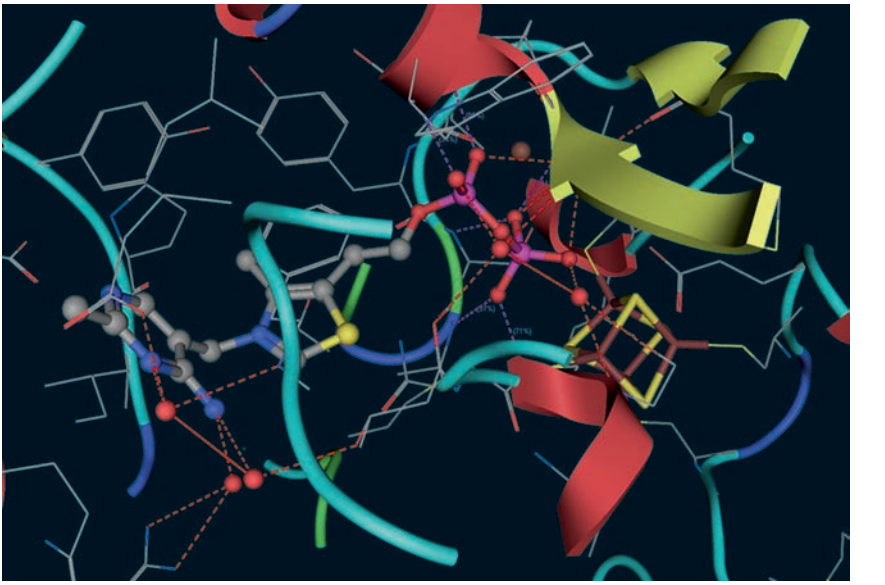

Figure 6.4 H-Bond network of the thiamine pyrophosphate cofactor of pyruvate ferredoxin oxidoreductase from *D. africanus*. Thiamine diphosphate is depicted in ball and stick representation, one iron/sulfur cluster is depicted in stick representation. The protein backbone is drawn in cartoon representation with secondary structure elements shown as red helical coils (α-helices) and yellow arrows (β-sheets). Amino-acid side-chains are drawn in wireframe representation. Water molecules participating in the H-bond network are represented as red spheres at the position of the water-oxygen atoms. H-bonds are drawn as dotted lines in red. (PDB-Code 2C3M).

no counterpart in higher organisms, this enzyme is highly promising as a drug target. So far, potent inhibitors of PFORs are not yet available. Current drugs that are effective against anaerobic protozoa by interfering with the PFOR-catalyzed reaction are the 5-nitroimidazoles, including metronidazole and tinidazole, the drug family of choice for treating anaerobic protozoal infections [20]. However, the susceptibility of the parasites to these drugs is not caused by a direct inhibition of PFOR but is due to a reductive activation of the nitro group by the ferredoxin reduced during pyruvate oxidative decarboxylation to form reactive free-radical derivatives. Although the precise action mechanism of the radical derivatives is still unknown, drug-induced damage of DNA and membrane components are most likely responsible for the observed cytotoxic effects.

Methionine γ-Lyase (Sulfur Amino Acid Metabolism)

Methionine γ-lyase (MGL, methioninase, EC 4.4.1.11) catalyzes the simultaneous deamination and dethiomethylation of sulfur-containing amino acids to yield thiol compounds, ketoacids, and ammonia (Figure 6.5). The pyridoxal 5′ phosphate

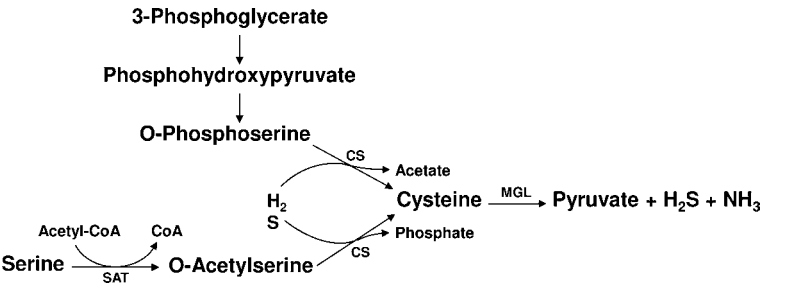

Figure 6.5 Pathways for cysteine biosynthesis in amitochondriate protozoa. CS, cysteine synthase; MGL, methionine-γ-lyase; SAT, serine *O*-acetyltransferase. *T. vaginalis* that lacks a SAT homolog is suggested to synthesize cysteine from phosphoserine.

(PLP)-dependent enzyme has been found in various anaerobic bacteria, plants and anaerobic protozoans, including *E. histolytica*, *E. dispar* and *T. vaginalis*, but was not detected in *E. invadens*, *T. foetus*, and *G. lamblia* [21, 22]. Unlike other organisms containing MGL, *E. histolytica* and *T. vaginalis* habor two isotypes of this enzyme, with different biochemical properties and biological functions. These enzymes also lack activity toward cystathionine, consistent with the absence of a transsulfuration pathway in anaerobic protists. Recently, one of the MGL isoforms from *E. histolytica* (EhMGL2) and the bacterium *Pseudomonas putida* were crystallized and their three-dimensional structures solved [23, 24].

These data have supplied detailed information on the substrate- and PLP-binding pockets of the enzyme that may be useful for a structure-based design of drugs targeting MGL (Figure 6.6). MGL can be completely inactivated by propargylglycine, but this compound was not able to block the growth of *E. histolytica* under *in vitro* culture conditions [21]. Trifluoromethionine, a methionine analog specifically activated by MGL, was shown to be highly toxic to *T. vaginalis* and *E. histolytica in vitro* and also cured experimental infections of both parasites in mice and hamster models [21, 22]. These observations suggest that trifluoromethionine may be a lead candidate for a novel class of drugs with potential against a range of bacteria and anaerobic protozoa.

Serine *O*-Acetyltransferase (Sulfur Amino Acid Metabolism)

Anaerobic protozoa lack glutathione and its recycling enzymes and use instead cysteine as the major reducing agent and antioxidant. In common with bacteria and higher plants, these parasites are capable of synthesizing cysteine from serine and inorganic sulfide, while higher animals generate this thiol from methionine and serine using the trans-sulfuration pathway. The former biosynthetic process involves a two-step reaction in which either *O*-acetylserine or *O*-phosphoserine is synthesized following the transfer of the alanyl moiety to inorganic sulfide catalyzed by cysteine synthase (CS; Figure 6.5). In *E. histolytica*, most bacteria and plants, the physiological

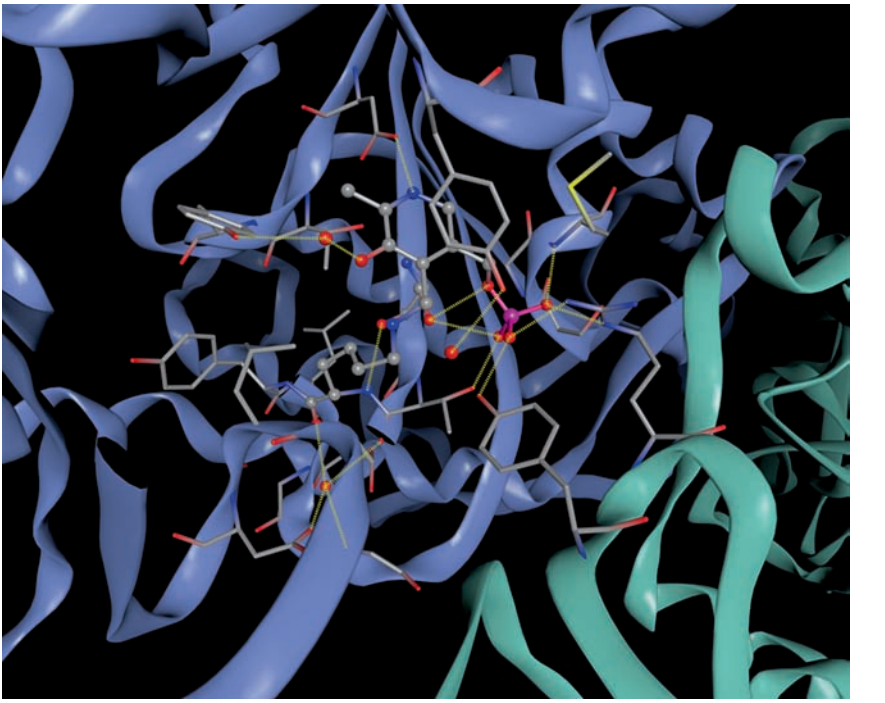

Figure 6.6 Complex H-bond network of PLP and two neighboring protein chains of L-methionine γ-lyase from *P. putida*. The protein chains are depicted in ribbon representation (chain A blue, chain B green). PLP is drawn in ball and stick representation, amino acid side-chains in stick representation and water molecules participating in the H-bond network are drawn as red spheres at the position of the water oxygen atoms. H-bonds are drawn as yellow dotted lines. (PDB-Code 2O7C).

substrate of CS is *O*-acetylserine that is produced from serine and acetyl-CoA by L-serine *O*-acetyltransferase (SAT, EC 2.3.1.30; Figure 6.5) [21]. This reaction is suggested to be a key point of regulation for cysteine biosynthesis due to its feedback inhibition by cysteine. A second type of SAT not sensitive to cysteine inhibition is present in plants [25]. Unlike in bacteria and plants where SAT forms a heterodimeric complex with CS, SAT (SAT1) in *E. histolytica* lacks an interaction with CS. Genome analyses have revealed two additional SAT isoforms in this parasite that differ in structure and physicochemical properties from SAT1 [21]. *T. vaginalis* appears to possess a sulfur amino acid metabolism similar to that of *E. histolytica* but lacks a SAT homolog and is thus unable to generate *O*-acetylserine [26]. As an alternative, cysteine biosynthesis in this protist is likely to involve *O*-phosphoserine as shown for the hyperthermophilic archaeon *Aeropyrum pernix* (Figure 6.5) [27].

In this pathway, *O*-phosphoserine may be provided from glycolytically derived 3-phosphoglycerate with phosphohydroxypyruvate as an intermediate. The three-dimensional structure of bacterial SATs was determined and their substrate and

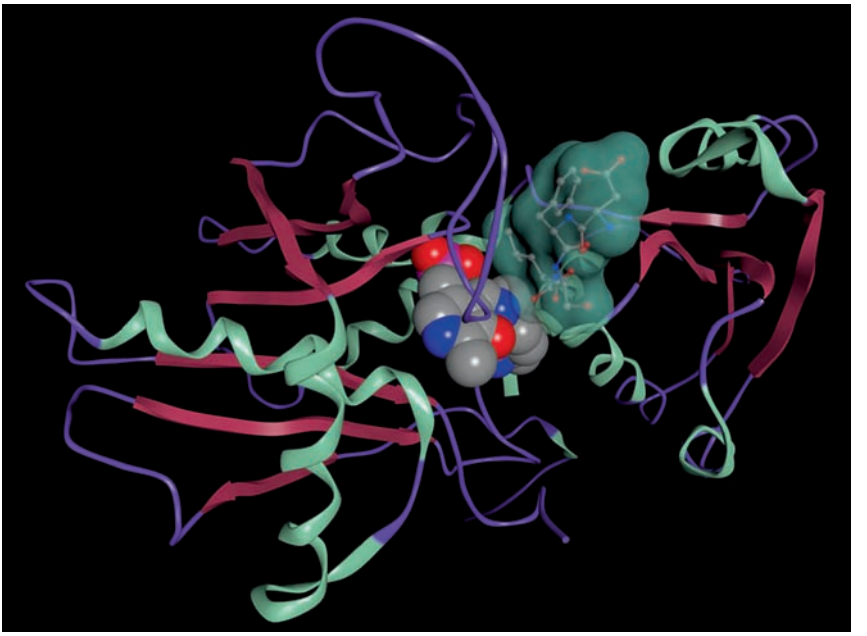

Figure 6.7 Cysteine synthase from *M. tuberculosis* crystallized with the inhibitory peptide DFSI. The protein chain is depicted in cartoon representation, coloring representative of secondary structure elements. The PLP cofactor in the active site is drawn in CPK representation. The four-residue inhibitory peptide is drawn in ball and stick representation overlaid with the solvent accessible surface (translucent). (PDB-Code 2Q3C).

inhibitor binding sites were defined [28]. The enzyme is a 175 kDa hexamer made up of two trimeric structures that appear to interact with each other at N-terminal ends. Recently, three multi-ring heterocyclic compounds identified by virtual screening of a chemical library against *Escherichia coli* SAT were found to block the proliferation of *E. histolytica in vitro* at low drug concentrations. As these substances did not affect a mammalian cell line, they may be of further interest as potential lead candidates for the development of novel anti-amoebic agents [29].

Cysteine Synthase (Sulfur Amino Acid Metabolism)

Cysteine synthase (CS, *O*-acetylserine sulfhydrylase, EC 2.5.1.47) is a PLP-dependent enzyme that catalyzes the last step in the cysteine biosynthetic pathway operative in most eubacteria, archaea, plants, and some parasitic protozoa (Figure 6.5). The structural changes and mechanism of catalysis underlying the enzymatic reaction have been characterized extensively by kinetic studies, site-directed mutagenesis, and X-ray analyses [30–32]. The reaction follows a ping-pong mechanism in which acetate is β-eliminated from acetylserine to form a α-aminoacrylate intermediate to which

the hydrogen sulfide is added resulting in the generation of cysteine. In bacteria and plants two CS isoforms, CS-A and CS-B (also denoted CysK and CysM) are found exhibiting characteristic differences in their substrate specificity. CS-A uses mostly hydrogen sulfide as sulfur source, whereas the CS-B type tends to accept larger thiol-carrying compounds as substrates. The crystal structure of various A-isoforms from bacteria, archaea, and plants, and two structures of bacterial CS-B isoforms have been determined [30–32] (Figure 6.7). The *E. histolytica* genome appears to encode three genes for CS, two of which only differ from one another by three nucleotides [21, 33].

Genetic complementation studies with a CS-A deficient *E. coli* strain suggest that all *E. histolytica* CS isoforms are functional as CS-A. *E. dispar* possesses CS isoforms similar in structure to the *E. histolytica* enzymes. *T. vaginalis* relies also upon CS for cysteine biosynthesis, and the analysis of the trichomonad genome revealed six gene copies for this enzyme [21, 26]. One of the isoforms (TvCS1) was produced as a recombinant enzyme and biochemically characterized [26]. These studies, together with the finding of the absence of SAT in this parasite, provide evidence that TvCS1 is likely to use *O*-phosphoserine instead of *O*-acetylserine as a substrate in common with the CS from the hyperthermophilic archaeon *A. pernix* and some bacterial CS-B isoforms [27]. These enzymes possess a conserved loop with a conserved basic

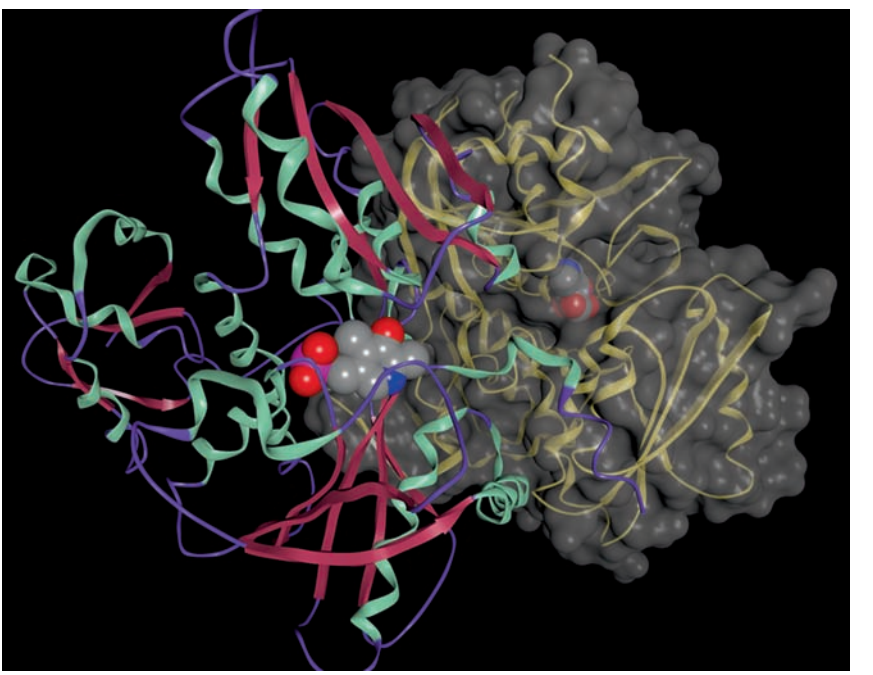

Figure 6.8 *O*-phosphoserine sulfhydrylase from *A. pernix*. Chain A is depicted in cartoon representation, coloring representative of secondary structure elements. Chain B is drawn in cartoon representation and overlaid with the solvent accessible surface (translucent). PLP is drawn in CPK representation. (PDB-Code 1WKV).

residue near the active site that appears to confer the ability to use *O*-phosphoserine as shown by site-directed mutagenesis experiments. The crystal structure of CS from *A. pernix* was recently determined and characterized extensively (Figure 6.8) [27]. This CS was considered a new enzyme that should be named *O*-phosphoserine sulfhydrylase (newly assigned as EC 2.5.1.65). Certain triazole, pyrazole, and tetrazole compounds, some of which are reported inhibitors of bacterial and plant CSs, were shown to be cytotoxic against *E. histolytica* cultures *in vitro* but their mechanism of action in the parasite was not investigated [21].

Trypanosomatid Protozoa

Most of the knowledge about the biochemistry and cell biology of trypanosomatid protozoa has derived from studies on *Trypanosoma brucei, T. cruzi*, and a few *Leishmania* species. This work has uncovered a variety of biochemical features unique to these organisms. Two components of these unusual metabolic routes that are indispensable for survival of the parasite are discussed in more detail.

Trypanothione Reductase (Thiol Metabolism)

Trypanosomatids possess a unique thiol metabolism, in which trypanothione, N^1,N^8-bis(glutathionyl)spermidine, is used to perform similar protective and regulatory roles as glutathione (GSH) in most other cells (see also Chapter 12). The novel thiol is synthesized in a stepwise, ATP-dependent reaction from spermidine and GSH by a monomeric trypanothione synthetase (EC 6.3.1.9) and maintained in its metabolically active dithiol form by the NADPH-dependent, flavin-containing trypanothione reductase (TryR, EC 1.8.1.12; Figure 6.9) [34, 35].

Genetic knockout and other studies have shown that the enzyme is essential for survival of trypanosomatids both *in vitro* and in the mammalian host [37]. Comparison of the *T. cruzi* TryR and the homologous human GSH reductase crystal structures revealed significant differences between their active sites that explain the selectivity shown by the homologous enzymes for their cognate substrates [35]. Various TryR inhibitors have been discovered by high-throughput screening of compounds from commercial libraries or by structure-based inhibitor design (Figure 6.10). Some of

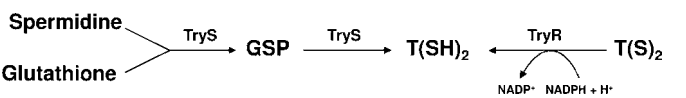

Figure 6.9 Trypanothione metabolism in trypanosomatid protozoa. GSP, glutathionylspermidine; $T(S)_2$ and $T(SH)_2$, oxidized and reduced forms of trypanothione, respectively; TryR, trypanothione reductase; TryS, trypanothione synthetase.

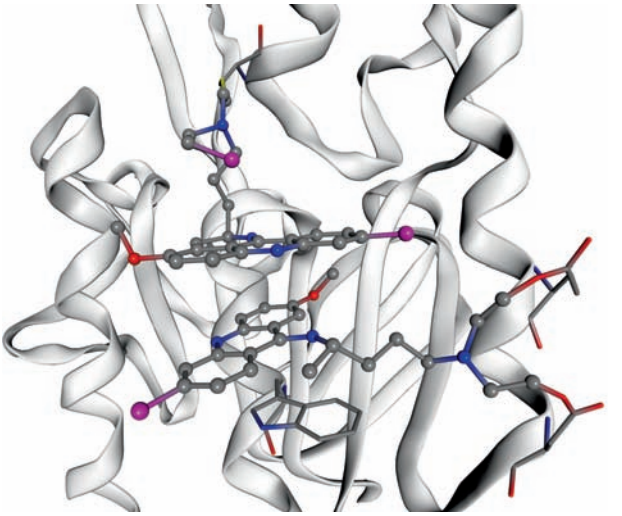

Figure 6.10 Active site of *T. cruzi* trypanothione reductase in complex with the inhibitor quinacrine mustard. The protein chain is depicted in ribbon representation. The inhibitor quinacrine mustard is drawn in ball and stick representation and relevant trypanothione residues are drawn in stick representation. Two molecules of the inhibitor are covalently bound to residues in the trypanothione binding site. The inhibitor molecules interact with each other through π-stacking effects and one inhibitor molecule interacts in the same way with TRP22 [36]. (PDB-Code 1GXF).

these compounds show high selectivity for TryR over mammalian GSH reductase and *in vitro* selective activities against trypanosomatids in the low micromolar range [38]. Other components of the trypanothione system that have also been considered as potential drug targets, such as trypanothione synthetase, are discussed in a separate chapter in this book.

Trypanosome Alternative Oxidase (Energy Metabolism)

The bloodstream forms of *T. brucei*, the causative agents of African sleeping sickness and similar diseases in domestic animals, rely entirely on glycolysis for their energy production [2, 39]. In this process, blood glucose is used as the source of energy that is catabolized to pyruvate as the sole end product. Unlike in anaerobic glycolytic fermentations, in which redox balance is maintained by lactate or ethanol formation, in *T. brucei* and some related species the reducing equivalents generated during glycolysis are shuttled via glycerol-3-phosphate to an unusual type of respiratory chain located in the inner membrane of their single mitochondrion [39, 40] (Figure 6.11).

The holoenzyme complex is composed of a flavin-containing glycerol-3-phosphate dehydrogenase linked through ubiquinone 9 to a glycerol-3-phosphate oxidase, named trypanosome alternative oxidase (TAO). Electron transfer from glycerol-3-phosphate to TAO and subsequently to oxygen is terminated with the formation of

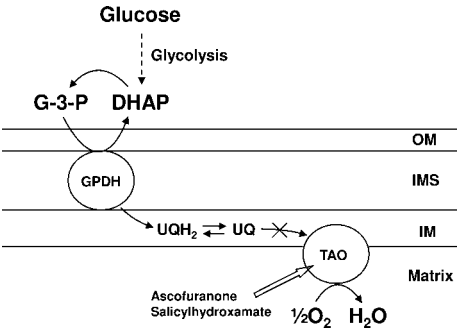

Figure 6.11 The mitochondrial electron transport system in the bloodstream forms of *T. brucei* (modified after [38]). Ascofuranone and salicylhydroxamate inhibit electron transfer from ubiquinol to oxygen. DHAP, dihydroxyacetone phosphate; G-3-P, glyceraldehyde-3-phosphate; GPDH, glycerol-3-phosphate dehydrogenase; IM, mitochondrial inner membrane; IMS, mitochondrial intermembrane space; OM, mitochondrial outer membrane; UQ and UQH_2, oxidized and reduced forms of ubiquinone 9, respectively; TAO, trypanosome alternative oxidase.

water and is not coupled to ATP conservation. TAO is a 33 kDa diiron membrane protein with structural similarities to the alternative terminal oxidases identified in bacteria, fungi, plants, and some invertebrate phyla, but is not found in vertebrates [39]. In contrast to the cytochrome-dependent conventional mitochondrial respiratory chain, TAO is resistant to cyanide but sensitive to aromatic hydroxamates, such as salicylhydroxamate (SHAM), and the antibiotic ascofuranone [39]. Recent studies have shown that SHAM at low micromolar concentrations can kill bloodstream forms of *T. brucei* in culture, and repeated administration of ascofuranone could cure infections with *T. brucei* and the closely related *T. vivax* in mice [39, 41]. Recently designed novel compounds combining structural similarities to SHAM and ubiquinol were found to inhibit TAO more effectively than SHAM and blocked the growth of bloodstream *T. brucei in vitro* [42]. These findings suggest that TAO remains an attractive, selective target for the development of new treatments for diseases caused by *T. brucei* and related trypanosome species.

Apicomplexan Parasites

Apicomplexan parasites, such as *Plasmodium falciparum*, *Toxoplasma gondii*, and *Cryptosporidium parvum*, harbor a number of metabolic pathways that are essential for the survival of the pathogen but are not existent in the mammalian host thereby constituting attractive targets for new antiparasitic agents. One of these unusual features is the ability of the protozoans to synthesize chorismate (shikimate pathway), the key metabolic intermediate in prokaryotes, fungi, and plants for the biosynthesis of an array of aromatic compounds, including folate, quinones, and aromatic amino

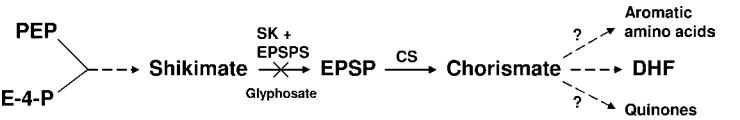

Figure 6.12 The shikimate biosynthetic pathway in apicomplexan protozoa. Phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E-4-P) are converted to chorismate via shikimate and 5-enolpyruvylshikimate-3-phosphate (EPSP) in a series of seven enzymatic steps. Biosynthesis of dihydrofolate (DHF) from chorismate consists of a series of four enzymatic steps. Broken arrows symbolise multi-step enzymatic reactions. Question marks indicate pathways that remain to be demonstrated in apicomplexan protozoa. Glyphosate is a broad spectrum herbicide that causes shikimate accumulation through inhibition of EPSP synthase [42]. CS, chorismate synthase; EPSPS, EPSP synthase; SK, shikimate kinase.

acids (Figure 6.12). Another unusual metabolic aspect of various apicomplexans is the utilization of a mevalonate-independent route for the biosynthesis of isopentenyl pyrophosphate (IPP) which is the common precursor for the biosynthesis of isoprenoids, such as steroids, dolichols, and quinones. Apicomplexans form this compound within their non-photosynthetic plastid-like organelle (apicoplast) through the 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway that is also operative in most eubacteria, and in the plastids of algae and higher plants (Figure 6.14). However, it is absent in animals, fungi, the cytosol of plant cells, and in apicomplexans that have lost the apicoplast, such as *Cryptosporidium* spp. These systems alternately use the mevalonate pathway for isoprenoid synthesis.

Chorismate Synthase (Shikimate Biosynthetic Pathway)

The shikimate pathway consists of seven enzymes that catalyze the stepwise conversion of erythrose-4-phosphate and phosphoenolpyruvate to chorismate (Figure 6.12). In apicomplexans, this pathway has been reported to be cytosolic and the molecular arrangement of the enzymes appears to be the same as in fungi, including a pentafunctional enzyme complex (AROM protein), but different from that in bacteria and plants [43]. However, the function of the shikimate pathway in apicomplexan parasites other than in folate biosynthesis remains to be demonstrated. Chorismate synthase (CS, EC 4.2.3.5) catalyzes in the shikimate pathway the conversion of 5-enolpyruvylshikimate-3-phosphate (EPSP) to chorismate, the last common precursor in the biosynthesis of aromatic compounds (Figure 6.12) [44].

The reaction of CS is strictly dependent on reduced flavin mononucleotide (FMN) and involves the *anti*-1,4-elimination of the 3-phosphate and C-(6*proR*)-hydrogen from EPSP, both aspects which makes this enzyme biochemically unique in nature. While bacterial and plant CSs lack the ability to generate the reduced FMN cofactor, the so-called bifunctional CSs possess intrinsic NAD(P)H:FMN oxidoreductase activity to directly reduce the bound FMN. This class of enzymes is found in fungi and some free-living protozoa. Although the CS from apicomplexan protozoa is similar to the fungal enzyme, the presence of bifunctionality in CS from the parasites remains to be established. The mechanism of catalysis of CS was extensively studied

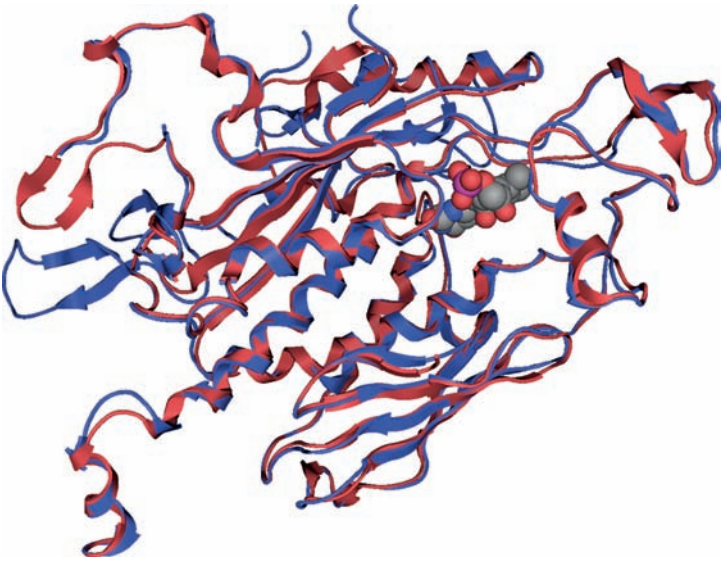

Figure 6.13 Homology model of the *P. falciparum* chorismate synthase. The 3D structure of chorismate synthase (Uniprot acc. O15864) was modeled by comparative homology modeling using Chemical Computing Group's software suite MOE. *Haemophilus pylori* chorismate synthase (PDB-code 1UM0) was used as template structure. The protein alignment of the two sequences revealed some inserts for the *P. falciparum* sequence which could not be modeled. Therefore, these areas are not depicted. Template (blue) and model (red) structures are depicted in cartoon representation. Secondary structure elements – α-helices and β-sheets - are shown as helical coils and arrows, respectively. The cofactor FMN from the template structure is depicted in CPK representation. Within the core region the model structure is in very good agreement with the template structure.

by using site-directed mutagenesis, substrate analogs, and high-resolution structure data. The crystal structures of bacterial and fungal CSs revealed β–α–β sandwich topology and a tetrameric arrangement of the molecule composed of two dimers [44–47]. Many of the conserved residues present in the primary sequence of the active site of CS show conserved conformations in all structures solved.

Structural and spectroscopic data suggest major conformational changes of the enzyme upon cofactor and substrate binding. Recently, the structure of CS from *P. falciparum* (Figure 6.13) was predicted on the basis of known CS structures and some of its properties described using bioinformatic tools [48]. A number of inhibitors of CS have been reported, including fluorinated shikimate analogs and a series of benzofuranone compounds [47].

1-Deoxy-D-Xylulose-5-Phosphate Reductoisomerase (Isoprenoid Biosynthesis)

The MEP pathway that consists of seven enzymatic steps starts with the condensation of pyruvate and glyceraldehyde-3-phosphate to produce 1-deoxy-D-xylulose-5-phosphate (DXP) by DXP synthase (Figure 6.14). The second enzyme of this route, DXP

Figure 6.14 The MEP pathway. Biosynthesis of 4-hydroxy-3-methyl-but-2-enyl diphosphate (HMBPP) from 2-C-methyl-D-erythritol-4-phosphate (MEP) consists of a series of four enzymatic steps. DMAPP, dimethylallyl diphosphate; DXP, 1-deoxy-D-xylulose-5-phosphate; DXR, DXP reductoisomerase; G-3-P, glyceraldehyde-3-phosphate; IPP, isopentenyl diphosphate. Fosmidomycin inhibits the reaction catalyzed by DXR.

reductoisomerase (DXR, EC 1.1.1.267), catalyzes in the presence of NADPH and a divalent cation the conversion of DXP to MEP by intramolecular rearrangement of the carbon skeleton of the DXP and a reduction step. The product, MEP, is a precursor for not only IPP, the key metabolite for all isoprenoids, but also the vitamins B1 and B6.

Several crystal structures of bacterial DXRs complexed with or without cofactors and/or substrates/inhibitors have been solved and the domains for substrate, cofactor, and inhibitor binding characterized [49, 50]. These data provide detailed insights into the structure and function of the enzyme that should assist the future design of structure-based inhibitors. Fosmidomycin and some structurally similar derivatives are the most potent inhibitors of DXR currently known [50–52] (Figure 6.15).

The phosphonohydroxamate compounds act as mixed-type inhibitors of DXR with IC_{50} values against the enzyme from *P. falciparum* and various bacteria at nanomolar concentrations. Fosmidomycin was also reported to inhibit the *in vitro* growth of malarial parasites and cure rodent malaria caused by *P. vinckei* and humans infected with *P. falciparum* [52]. Homology modeling of DXR from *Mycobacterium tuberculosis* and analysis of protein–ligand complexes showed that fosmidomycin tightly binds in the active site of the enzyme by several interactions including hydrogen bonding to the phosphonate moiety and coordination of the hydroxamate group with the enzyme-bound Mg^{2+} ion [51]. Recently, various attempts have been made to improve the antimalarial activity of fosmidomycin by chemical modification or by combining the compound with other drugs [53].

Helminths

Due to the closer evolutionary relationship with their vertebrate hosts, helminths can be assumed to possess a lower number of unique drug targets than parasitic protozoa. To date, only a few functional molecules have been identified in parasitic worms that do not exist in mammals. Other unusual systems, though showing homology to mammalian proteins, have diverged into novel functions under the driving force of adaptive evolution. Two examples for such systems are briefly reviewed, one being part of a unique type of anaerobic energy metabolism widely present in worm parasites and the other a chloride-gated ion channel that is specifically expressed in

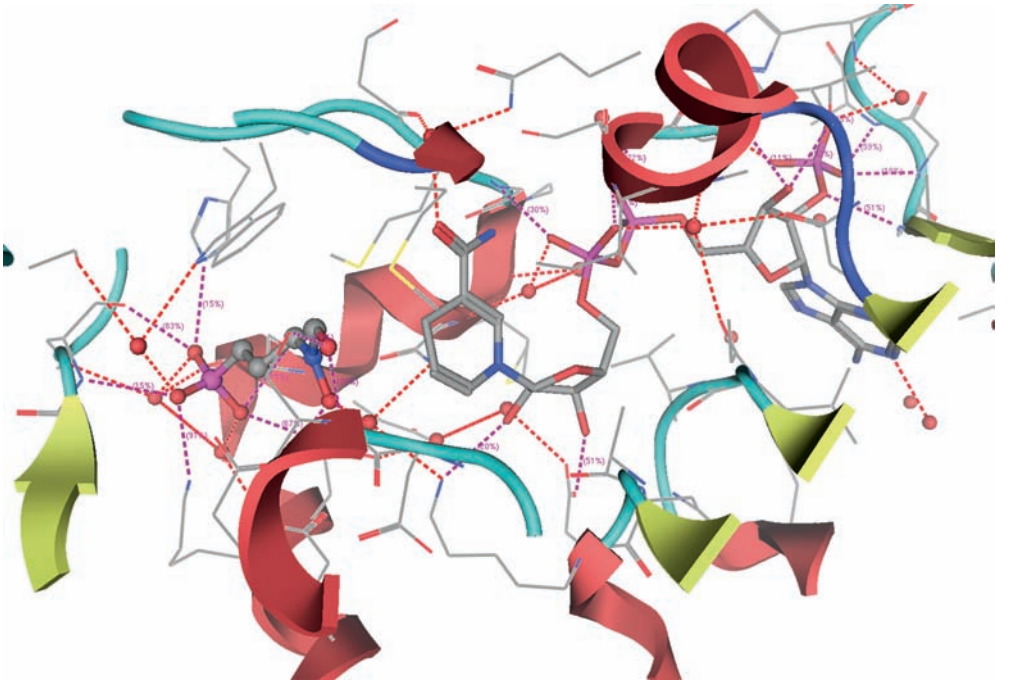

Figure 6.15 Complex H-bond network of fosmidomycin and NADPH in the binding site of 1-deoxy-D-xylulose 5-phosphate-2-reductoisomerase. Fosmidomycin is depicted in stick representation and NADPH is depicted in ball and stick representation. The protein backbone is drawn in cartoon representation with secondary structure elements shown as red helical coils (α-helices) and yellow arrows (β-sheets). Amino acid side-chains are drawn in wireframe representation. Water-molecules participating in the H-bond network are represented as red spheres at the position of the water-oxygen atoms. H-Bonds are drawn as dotted lines in red. (PDB-Code 2JCV).

nematodes and arthropods and has previously been identified as the molecular target for a number of most effective antiparasitic drugs currently known.

NADH-Fumarate Reductase (Anaerobic Energy Metabolism)

A striking feature of the metabolism of helminths, in particular of the adult stages, is the prevalence of anaerobic energy-yielding routes that are functional even if oxygen is available. A key component in these pathways is a mitochondrial electron transport system that serves to transfer reducing equivalents from NADH to fumarate as terminal electron acceptor and ends with the formation of succinate (Figure 6.16) [3, 54, 55].

This NADH-fumarate reductase is composed of mitochondrial respiratory complex I (NADH quinone reductase) and complex II (quinol fumarate reductase) and is coupled, like the conventional-type mitochondrial respiratory chain, to site 1 ATP

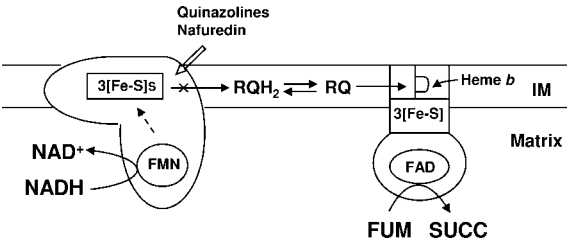

Figure 6.16 The NADH-fumarate reductase system of helminth mitochondria (modified after [53]). Quinazolines and nafuredin inhibit *A. suum* NADH rhodoquinone reductase (respiratory complex I). FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; FUM, fumarate; IM, mitochondrial inner membrane; RQ and RQH_2, oxidized and reduced rhodoquinone, respectively; SUCC, succinate.

generation. The functional difference between the fumarate reducing and the mitochondrial respiratory system of aerobic organisms is that electron transfer through complex II occurs opposite to the direction of succinate oxidation. An important factor determining this unusual reaction course is the requirement for rhodoquinone as an electron carrier instead of ubiquinone, the universal quinone species of oxygen-dependent mitochondrial respiration. The relatively low redox potential of rhodoquinone ($E^{\circ'} = -63$ mV) compared to that of ubiquninone ($+110$ mV) clearly favors electron flow towards complex II. In addition, distinctive features of the subunit components of complex II may assist to accomplish fumarate reduction. This includes the constituent cytochrome that was recently identified in *Ascaris* mitochondria and designated cytochrome b_{558} [54]. The relatively high redox potential (-34 mV) of this cytochrome appears to be more advantageous for electron transfer from reduced rhodoquinol (-63 mV) to fumarate than the homologous mammalian complex II cytochrome b_{560} (-185 mV). Helminth NADH-fumarate reductase activity is strongly inhibited by nafuredin, a lactone antibiotic, that was also shown to possess anthelmintic activity *in vivo* [54]. The compound was found to compete for the quinone binding site of complex I in a highly selective fashion, indicating that this domain structure in helminth complex I differs from that of its mammalian counterpart. NADH-fumarate reductase shows also remarkable sensitivity to various quinazoline derivatives possibly by interfering with the quninone reduction site of complex I [54, 55].

Glutamate-Gated Chloride Channel (Neuronal and Neuromuscular Transmission)

Glutamate-gated chloride channels (GluCls) are inhibitory ionotropic receptors that are found only in invertebrates [56]. These "Cys-loop" class of ligand-gated anion channels are closely related to the vertebrate $GABA_A$ and glycine receptors and are likely to possess a similar membrane-spanning pentameric subunit assembly (Figure 6.17). Each of the five subunits has a long, amino-terminal extracellular domain followed by four strongly conserved transmembrane regions and a large

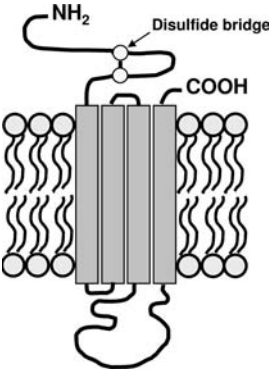

Figure 6.17 Schematic representation of the subunit topology of a ligand-gated ion channel with the four transmembrane loops and the cysteine disulfide bridge (modified after [55]).

cytoplasmic loop. Binding of glutamate to GluCls produces an increase in chloride ion permeability through channel opening resulting in rapid inhibitory actions within the invertebrate neuromuscular system. Two types of GluCl subunits involving various isoforms have been identified, designated GluClα and GluClβ [57, 58]. Seven such isotypes have been described for *Caenorhabditis elegans* and four for *Haemonchus contortus* [58]. These can assemble to multiple GluCl subtypes with differences in their pharmacological features and expression patterns among the target cells and worm species. GluCls are widely found within the nematode nervous system, including motor neurones, interneurones, and neurone commissures. In *C. elegans*, GluCl subunits (GluClα2A, GluClβ) have also been localized to the pharyngeal muscle cells, but the occurrence of these receptors on these cells in parasitic nematodes still needs definite clarification [58]. GluCls are the primary site of action for a number of pharmacological agents including some of the most effective antiparasitic drugs currently known, such as the macrocyclic lactones (avermectins, milbemycins) and certain phenylpyrazoles. In contrast to the fast-acting endogenous glutamate transmitter, the macrocyclic lactone antinematodal drugs exert their effect by slowly, but essentially irreversibly, opening GluCls upon binding to the receptor [57, 58]. The associated increase in chloride conductance causes a long-lasting hyperpolarization or depolarization of the neurone or muscle cell and thus inhibition of their further function. Results obtained from gene expression and mutation experiments suggest that the GluClα-type subunits appear to carry the binding site for macrocyclic lactones, involving a possible role for amino acid residue 299. The presence in nematodes of multiple forms of GluCls with varying pharmacological properties makes the mechanism of sensitivity against macrocyclic lactones in these organisms highly complex. Another drug acting on GluCls is the phenylpyrazole insecticide fipronil. This compound was shown to exert its major insecticidal effect through an open channel blocking mechanism that was also suggested to account for the selective toxicity of the drug observed between insects and vertebrates [59]. Elucidation of the subunit structures of the different GluCl isotypes and of similar ligand-gated anion channels known only from invertebrates,

such as the histamine- or serotonin-gated receptors, may offer opportunities for the discovery of new generations of highly selective antiparasitic and insecticidal drugs. A promising start for such research efforts is the recent report on the discovery of a new class of antinematodal compounds, known as aminoacetonitrile derivatives, that were shown to exert their potent activity by targeting a novel type of nematode-specific nicotinic acetylcholine receptor subunits [60].

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7
Lessons Learned from Target-Based Lead Discovery

*Michael Gassel, Jörg Cramer, Christopher Kern, Sandra Noack, and Wolfgang Streber**

Abstract

The discovery of new drugs is the mission of any innovative pharmaceutical company, whether human or veterinary. One way to develop new drugs is to systematically screen large compound collections through biochemical or cellular assays. Using knowledge about potential molecular mechanisms or targets, this target-based drug discovery is a well established process throughout the pharmaceutical industry. Whereas several successes support the feasibility of this approach, it has yet to be demonstrated that huge investments into this technology will create value, as a whole, for a company. Continuous learning and improvement is necessary. This chapter focuses on some of the improvements that have occurred during the past five years of target-based discovery within animal health research.

Drug Identification via Bioscreen and Target-Based Screen

All antiparasitics in the animal health market have been discovered in biological assay systems [1]. These systems rely, in part, on knowledge about parasite organisms and their life cycles and, in part, on serendipity, the explorative search for the unexpected. More than one decade ago new discovery approaches based on target screening were driven by the enormous knowledge that genomic science offered to the research community [2, 3]. Recent years, however, have clearly demonstrated that expectations in the new discovery approach were overestimated [4]. Many publications recognize this problem and propose remedies to increase success rates in modern drug discovery [5]. Such discussions reveal valuable general drivers for improvements. In modern parasitology, the predominant consideration must be the needs of the market, for example, a product that is safe, has good efficacy against a broad range of parasites, and has a long duration of effect. These needs have to be reflected and translated into objectives and suitable research processes. Investment in costly resources is only acceptable if new technologies can fulfil these needs.

Biological antiparasitic screening assays (bioscreens) are indispensable for antiparasitic drug discovery. A target-based discovery approach adds value to the discovery process in the following aspects:

- Target-based assays can reveal the full potential of a chemical class by dissecting many parameters involved in bioactivity (target activity, bioavailability, stability, to name only the most predominant ones) and establishing logical structure–activity relationships.
- Target-based assays have a minimal requirement of compound amount that is often about 1000-fold less than bioscreens, thus enabling access to a much larger chemical space.
- Recombinantly expressed target proteins can be used for screening in cases where cultivation of target organisms is difficult or even impossible in suitable amounts. This is especially the case with parasitic organisms.
- Recombinantly expressed target proteins from animal pathogen bacteria can provide assay systems that can specifically address disease-relevant mechanisms.

Visions and Objectives

Discovery of a new drug molecule requires a clear vision of what the molecule should do. From the beginning of our target-based screening, the drug candidate (Box 7.1)

Box 7.1: Definition of Lead and Drug Candidate

A Lead will:

- belong to a series of structures with a confirmed structure activity relationship (SAR)
- show the desired biochemical mechanism of drug action
- show no indication for genotoxicity
- be active in a relevant functional assay
- have adjustable physicochemical properties

A Drug Candidate will:

- have a distinct and defined structure
- show the desired biochemical and therapeutic mechanism of action in target animals
- be effective through the desired route of administration at a feasible dosage
- be safe and provide a therapeutic window
- guarantee a desired onset and duration of action
- qualify for large-scale preparation and indicate economic success.

has been the main objective of our discovery unit. The criteria for a drug candidate have to be carefully defined, while also being continuously refined over time. The drug candidate is not the result of the screening process alone, regardless of how sophisticated this process is. Instead, identification of a drug candidate also requires an optimization step based on synthetic variations of the molecule. Only an efficient combination of biological profiling steps and chemical derivations enables us to fulfill unmet biological needs from undiscovered chemical space.

The goal of the preceding process is a compound with well characterized properties that enable optimization leading to a drug candidate. This compound therefore is called a lead and the process is called lead discovery. Criteria for the lead compound have to be as carefully defined as for a drug candidate (Box 7.1). The main question for this task is how the criteria for a drug candidate translate into the criteria for a lead compound. The answer to that question, however, depends on the answer to a second question: which properties of a compound can be successfully improved during the lead optimization process versus which properties should be subject to selection during the screening process? Many theoretical considerations for improving molecules can be incorporated from past successful experiences. But rarely do we see objective data for overall probabilities within improvement processes, including those processes that failed. As a consequence we make an effort to learn from our own screenings and optimizations. We also make an effort to learn from concurrent experiences of others discussed during conferences and personal communication (The Society for Biomolecular Sciences, 36 Tamarack Avenue, # 348, Danbury, Connecticut 06811, USA). Unfortunately, many questions specifically related to explorative animal health research cannot be deduced from common knowledge.

Our discovery process started with a vision of having the right technology and sufficient knowledge from the post-genomic era available for translating our objectives into the right selection and optimization cascade. This vision became manifest in several working hypotheses. However, some of these were too optimistic, or at least resulted in insufficient success probabilities. In the following paragraphs we highlight three hypotheses that have undergone a significant change during our learning process. Certainly these, as well as other working hypotheses, will continue to be further evaluated in the future.

Validation of Targets

Initially we assumed that any molecular target that is validated by genetic means and functional evidence can be druggable, that is, can be modulated by a small-molecule drug. Druggability of a target [6] in the target-based approach is linked to the properties of the target's potential drug-binding site [7, 8]. These intrinsic properties have a major influence on the success probability [9]. We observed, however, some genetically validated targets yielding compounds with high *on-target* activity and good physicochemical parameters, but no biologically active molecules. As a consequence, we concluded that the genetically validated status of a probable target alone is not sufficient to choose a target as the starting point for screening.

We consequently turned to chemically validated and druggable targets. The electron transport chain of parasites, for example, proved to be druggable (see Chapter 19). Highly druggable targets not only increase the probability of finding biologically active lead compounds, but also create the opportunity to explore a large chemical space with synthetic variations to improve a spectrum of properties.

In accordance with this strategy, we invested significant effort in determining the druggability and chemical validation of a target before we engaged in costly screening and optimization. A molecular target that has been chemically validated by a known targeting compound has first priority for target-based drug discovery. Additional genetic validation enhances the success by insuring that the validated targeting compound really acts on the assumed target [10]. Genetic validation alone also is a valid starting point for a target if no targeting compound for chemical validation is available. In that instance, the validation should be completed by chemical validation as soon as suitable targeting compounds are identified for the assumed target. The resulting portfolio contains highly validated targets and promising new targets to balance the risks with the opportunities (see Chapter 5).

Toxicity of a Lead

In human drug development, lack of clinical efficacy and unexpected adverse effects account for the majority of all drug failures [11]. In animal drug development, clinical efficacy can be addressed early by animal studies, but toxicity is one major hurdle on the way towards a drug candidate. We initially assumed that the possibility to optimize this property at a later stage is rather low and that it therefore has to be addressed as early as possible by selecting compounds during the hit-to-lead process. We also believed that molecular and cellular assays have a high value for predicting toxicity.

Toxicity is like any other drug action mediated by molecular drug targets. On the molecular level, low toxicity is equivalent to a low affinity for the corresponding targets, or in other words, high selectivity for the desired drug targets. Initially we designed our screening cascade with the ambitious goal to achieve high selectivity by filtering high-throughput screening hits using counterscreens based on the target's mammalian ortholog. With this stringent filter we experienced a dramatic reduction in the number of compounds that successfully passed all our criteria, especially those for potency and bioavailability. Pharmaceutical research for human disease has vast experience concerning selectivities and their improvement by target-accompanied testing of compounds during a synthetic optimization process. We therefore defined on-target selectivity as just one of several parameters that contribute to our decision for a lead compound (Figure 7.1).

Toxicity and side-effects (adverse drug reactions, ADR), may stem from interactions with secondary targets or other unknown targets [12]. Some of these targets, like hERG, can be addressed using molecular assays [13]. Some basic metabolic functions, like respiration, can be addressed using cellular assays [14]. Such assays are able to pinpoint possible reasons for toxicity. But our drugs finally have to be safe in

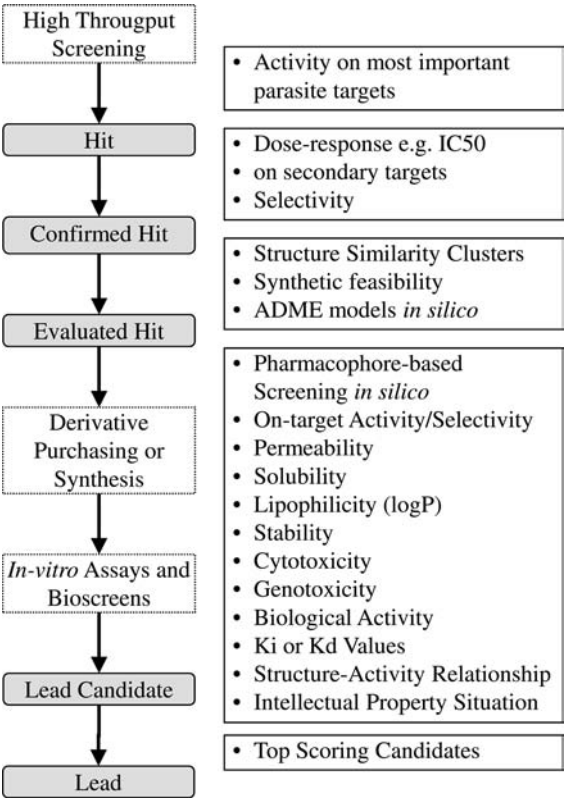

Figure 7.1 Main lead discovery workflow and lead properties.

complex organisms where many more mechanisms can account for ADRs. Single molecular and cellular safety assays are unable to predict the probability of failure in the preclinical and clinical phases [15]. Therefore, we do not use *in vitro* safety assays as criteria for selection, but rather we use them for detection of possible toxicity mechanisms. Early detection of a toxicity mechanism can guide the subsequent optimization process. If the toxicity mechanism is not associated with a strictly required feature of the molecule, there is a chance to separate toxicity from efficacy during the lead optimization phase. An appropriate animal model however is a prerequisite for this task. Such an animal model is usually not available during the hit-to-lead process.

Another possibility to handle ADRs is by the identification of potential modes of action, using molecular pharmacology profiles that have been generated by human research initiatives [16]. These profiles could guide lead optimization on a successful path to avoid later problems. Recently we have seen that such molecular pharmacology profiling can create new opportunities for animal health drugs [17]. This approach is developing and needs more experimental validation before the relevance for animal health can be judged.

Despite the foregoing efforts, the toxicity of a drug is still a major cause of failure. Molecular assays and cellular models should be used only to identify potential liabilities of lead candidates and to prioritize them for further optimization. Animal models and elaborate *in vitro* pharmacology profiles, including metabolism studies, should be used to support the lead optimization process, resulting in a drug candidate with a suitable therapeutic index.

Achieving Biological Activity

We assumed that biological activity of a molecule can be easily achieved by synthetically optimizing on-target activity. Biological activity in this context means the ability of a molecule to achieve a desired effect when applied to a whole organism as represented by model systems, like endo- or ecto-parasites or pathogen bacteria cultivated *in vitro*. Two observations recently formed our opinion concerning the chances to achieve such biological activity by synthetic modification of hits that stem from on-target discovery:

The first observation is based on historic experiences with target-based research projects wherein the majority of hits could not be optimized to achieve biological activity. This experience is shared between animal health and agrochemical research. Both areas are comparable because they deal with similar organisms, like arthropods, nematodes, fungi, and bacteria. Our in-house data clearly demonstrate that high on-target activity is the key to achieving biological activity. For example, within compound classes acting on the respiratory chain as an antiparasitic target, we see a clear correlation between activity on the target and activity in a parasite bioassay. But high on-target activity alone is not sufficient to achieve antiparasitic activity. This is true with several other compound classes that we identified by screening. Synthetic optimization, without knowledge of other important parameters, is prone to failure.

This observation motivated us to do an in-depth analysis of in-house screening data in order to search for further key parameters that are crucial for biological activity. At that time we had screened around 130 000 compounds against several parasitic organisms in bioassays. We correlated the results of these screenings with calculated molecule descriptors and predicted physicochemical parameters. This was impeded by the fact that our screening library already fulfilled drug-like properties as described further below in this chapter. This challenge, however, guaranteed that we would not reveal the same rules again. As a result of the analysis, we discovered a set of descriptors that tend to have a major influence on bioactivity. This set of descriptors is closely linked to the hydrophilic or polar properties of a molecule. The main components are:

- the number of hydrogen donors
- the number of hydrogen acceptors
- polar surface area (PSA)
- polar volume
- parameters describing the distribution of polarity within the molecule.

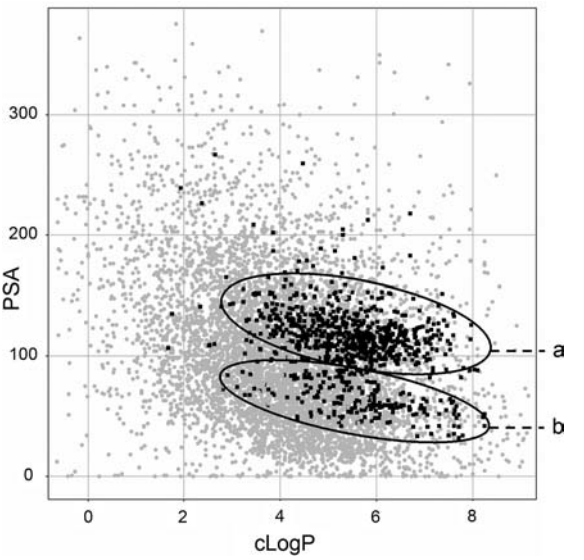

Figure 7.2 Molecular parameters PSA and calculated logP as developed during the course of a typical lead optimization project (black squares) and as covered by a random screening library (gray squares). The plot shows that each of the structural subfamilies (a or b) covers a broader range of calculated logP than of PSA. Parameters were calculated using SYBYL 7.3 (Tripos International, St. Louis, Missouri, USA).

In summary, molecules possessing the smallest and most concentrated polar surface components exhibited the greatest potential for biological activity. We found this to be generally applicable to all the tested molecules, including the subset that had been identified by their on-target activity. After a retrospective analysis of lead optimization projects, we realized that parameters describing polarity like PSA [18] are changed to a much lesser extent than other parameters like molecular weight and calculated logP [19] (Figure 7.2).

As a consequence of our findings, we postulate that the potential for biological activity has to be addressed before entering an extensive optimization process. Careful consideration of molecule descriptors during selection of screening compounds and hits can increase success probabilities, as well as testing on cells and whole organisms as early as possible. The knowledge that comes with compound classes already active in other biological systems is also helpful.

Target Selection and Screening Workflow

Like most pharmaceutical research, we focus on dedicated disease areas: anti-infectives, endoparasiticides, and ectoparasiticides. When we started in 2000, no

experience in target-based screening for animal health was available in-house. Therefore, our focus was on establishing target-based screening from scratch. Although the process *per se* is comparable to that of the human pharmaceutical sector, there were a number of challenges of the animal health business that required adaptations of the workflow. For example, little information about genes and genomes of the most important animal parasites like ticks, fleas, and helminths was available in the scientific community. As a consequence, no assays and cell lines for target proteins were established. Therefore, all our screening projects started with a target discovery phase that implemented fishing for genes, cloning the genes, demonstrating functionality, and developing assays which were robust and suitable for high-throughput screening (HTS) [20]. The following screening phase comprises primary and secondary screening, hit verification, and hit confirmation. These steps are all filtering processes separating "good" molecules from "bad" molecules. Whereas this general workflow did not change over time, we introduced significant changes concerning the complexity and the knowledge content of the single lead discovery steps.

Early screening projects started with a screening campaign on one ortholog of the target protein of choice, mostly derived from the model organisms *Drosophila melanogaster* and *Caenorhabditis elegans*, because of the absence of proteomic and genomic information of parasitic target organisms. Soon secondary screening filters were introduced using assays with target proteins isolated from our parasitic target organisms (Box 7.2). With this approach, we studied only the compounds most active on one model organism target rather than proceeding with the compounds having the broadest action on target organisms. This led to severe compound attrition in the course of the screening cascade. Over time, however, genomic information on target organisms became available for the recombinant production of target proteins and screening technology became less of a bottleneck for the lead discovery workflow. This progress allowed a much broader screening approach, starting with several orthologs of parasites or bacteria. Usually we perform primary screens on the targets of choice derived from two relevant parasitic or bacterial species and, if applicable, from one host animal. If compound resistance [21] has been reported and the target variants responsible for these are known, we include these variants in the primary screening. The goal is to arrive at an early impression of the profile of a compound as to its activity and selectivity and to find additional chemical classes. We are now rather working in a fashion as phrased by Mason: "Worry about profile, don't worry about potency" [22]. By specifically addressing the targets in the parasitic and infective organisms, we avoid the probable disconnect between compound activities on-target and biological assay systems. Model organisms are highly problematic when the evolutionary distance to

Box 7.2: Parasites and Infective Bacteria Used for Isolation of Target Genes and Proteins

- Endoparasites: *Haemonchus contortus, Ascaris suum, Ascaridia galli*
- Ectoparasites: *Ctenocephalides felis, Boophilus microplus*
- Bacteria: *Mannheimia haemolytica, Pasteurella multocida*.

target organisms is large, as observed for instance between *C. elegans* and *Ascaris suum*. If necessary, different subunit combinations are also screened. The challenge for the latter topic is to find the right balance between effort and benefit.

The chance of a lead compound to become a drug candidate is not only dependent on its on-target activity and selectivity. Similarly important is its interaction with complex biological systems, consisting of other targets, pathways, membranes, cells, organs, and whole organisms. These off-target properties mainly affect bioavailability and toxicity and together with on-target activities effect the desired biological activity. The last phase of a lead discovery project (also called the "hit-to-lead process") is therefore dedicated to these properties. It is characterized by less filtering and more broadening of the knowledge about chemical classes of hits. The need for better lead compounds led us to introduce more assays and more selection criteria for lead candidates that account for these properties. We developed *in vitro* assays for physicochemical properties responsible for bioavailability and additional tests based on whole cells or relevant bacteria grown under specific conditions to reflect the situation in the host and to simulate the aimed mode of action. These activities have the goal of increasing the probability to identify compounds that are active both on-target and in biological systems. In addition to increased selection assays, it became important to purchase and synthesize more derivatives of hit compounds in order to better evaluate the chances and pitfalls of the following optimization process, including synthetic feasibility (Figure 7.1).

Recent developments in discovery research motivated the physiology-based discovery approach where pharmacologic activities provide the starting point for novel therapies. Currently, we are starting to test compounds that have previously shown moderate antiparasitic activities systematically on molecular targets validated for animal health. The goal is to identify the mode of action of these compounds. As their biological activity alone is not sufficient to start a lead qualifying process or a lead optimization, we hope that knowledge about possible targets and modes of action can qualify these compounds for a successful optimization. Additionally, it opens the possibility to validate targets chemically that were previously identified by genetic validation.

All choices concerning the screening strategy have to be taken at the very beginning of the project and have to reflect the lead objectives. But with constant improvement in knowledge and experience, objectives and methods can change manyfold during the course of a long discovery process. Therefore it is necessary to constantly monitor these prerequisites and to adapt workflows concurrently, in order to keep the focus on the paramount objective: to reach the drug candidate.

Compounds and Libraries

Without any doubt compound libraries form an essential prerequisite for target-based screening in industrial drug discovery. The setup and maintenance of this asset

has gone through several phases and paradigms since high-throughput screening was first established about 20 years ago. Overall, the need for a more efficient discovery of active molecules has driven development [23].

Library Design for Effective Discovery

We usually screen about 130 000 compounds, which generally results in a hit rate between 0.1% and 3.0%, depending on the type of target and assay format. As outlined before, the follow-up process from a hit to a lead compound is strongly determined by technical feasibility. The capacity to evaluate, select, and optimize up to 2000 structurally diverse hits continuously faces bottlenecks. An effective lead discovery depends on the challenging combination of throughput performance and quality of the resulting information sufficient to enable educated decisions. The output of lead compounds in terms of quality and quantity is strongly dependent on the input of the screening library. Therefore, careful design of the screening library is crucial for success [24, 25]. In addition, continuous changes in research objectives demand significant adjustments, not only in the discovery process but also in library design.

When random screening was first conceived, it was assumed that lead compounds for a variety of biological targets could be discovered using a large variety of compounds [26, 27]. Different routes to this variety of compounds were followed within the community [28]. One route was to create diversity by variation of a limited number of core molecules. This concept was inspired by the evolutionary combination of amino acids within proteins, especially antibodies, and was realized by combinatorial chemistry resulting in distinct and relatively dense, but limited, chemical spaces. The second route was to create variety by high diversity or dissimilarity. This approach is represented by chemically diverse compound collections [29, 30]. Having the high diversity of antiparasitic and anti-infective molecular targets in mind, we clearly favored the second approach, as did most of the large pharmaceutical companies. Diversity was calculated using a BCUT matrix analysis of the compound libraries under default conditions (DiverseSolutions 6.3.2, University of Texas at Austin; distributed by Tripos International, St. Louis, MO 63144, USA). The past decade, however, taught us that pure chemical diversity does not automatically represent biologically relevant chemical space [31–35]. The biologically relevant chemical space instead is constrained by parameters determining the fate of compounds within living organisms. We determined our chances of finding biologically active molecules could be significantly increased if these constraints could be taken into account within a screening collection. Our studies, in fact, suggest that distinct calculated descriptors for drugs can be used to enrich potentially biologically active molecules within the available chemical space [36]. A set of rules derived from in-house biological active hits can be applied to select compounds for the whole anti-infective and antiparasitic research area. This approach was pioneered in human pharmaceutical research by Lipinski as the *Rule of 5* [37], and was demonstrated for the agrochemical area by Syngenta as the *Rule of 3* [38, 39]. A more focused selection can be achieved when rules are individually derived from and applied

to distinct target organisms. Tice [40] highlighted the importance of certain surface area parameters for herbicidal and insecticidal activity. Similarly we identified different requirements for antibacterial, endo-, and ectoparasiticide activity (see Chapter 9).

We defined and applied constraints using ranges for polar surface area (PSA), H-bond acceptors and donors, molecular weight, calculated logP, and the distribution of the molecule polarity as filtering criteria for new compounds. The number of H-bond acceptors (ideal >2) and the PSA (70–160 $Å^2$) has a significant impact. We also determined that the commercially available CACO-2 [41] model within the Volsurf software [42] works well as an initial alert system for bioavailability. Moreover, we considered the synthetic complexity of the ligands, a combined view on rotatable bonds, the molecular weight, and stereocenters of a molecule. Complexity is used as a rough alert for long process timelines because re-supply of complex molecules and their derivatives might be difficult. In addition, we check the absence or presence of an in-house defined set of substructures in commercial libraries that we believe to be structurally relevant, based on synthetic considerations and observations. In a next step, the diversity analysis addresses the structural novelty or similarity, balancing between chemical accessibility and biological relevance. All of these property, structure, and diversity filters direct compound acquisition.

Unfortunately, we are not aware of any comprehensive *in silico* predictions for additional favorable compound properties, like solubility and chemical stability. Therefore, we can only rely on statistical serendipity and operational measures to keep these factors within an optimal range for a screening library.

Focused Libraries

In addition to a universal screening library designed by general rules, the use of focused libraries has also been considered to enhance the efficiency of lead discovery. A focused library is created by selecting compounds that fit a structure model [23, 43]. This method can only be applied when the mechanism of action and significant binding features are known. The same applies to using pharmacophores to exclude potentially unwanted drug effects like toxicity [44], metabolism [45] or multidrug resistance [46]. Both methods, however, are inadequate for generating a primary screening library that is intended to address all sorts of molecular targets, target species, and host species.

Pharmacophore-based focused libraries are best applied within the hit-to-lead process to populate the chemical space around identified hit structures with bioisosteric chemical types targeting the same binding site [47]. Our library design and library size rarely can rely on sufficient structural information of overlapping ligands or protein ligand complexes. In the absence of target-protein or pharmacophore structures, focused libraries designed for a whole target class or mechanism, for example, proteases, are frequently utilized as an approximation [48]. However, their effectiveness for discovery has never exceeded random selection in our studies.

Apart from structural design and target focus, other features of focused libraries might add value to lead discovery when used for a primary screening approach. Some libraries provide consistently accessible chemistry, while others provide structural exclusivity or molecular properties favorable for bioavailability. Libraries consisting of drugs already approved for treatment of human or veterinary diseases might bring a bundle of knowledge that is useful for further development. For example, known pharmacokinetic and safety behavior could effectively guide early clinical trials. As no single library can comprise all of these features, we pursue a mixed approach to take advantage of present and future opportunities.

Library Attrition

There are usually two sources of library attrition: one is routine consumption during a discovery process, and the other is decomposition of compounds.

In general, HTS libraries in antiparasitic drug discovery for animal health encounter higher substance consumption rates than those in other drug discovery activities. This is due to the high number of tests on molecular and whole-organism targets. To avoid the attrition of large library parts, a permanent replacement either by internal synthesis or acquisition from external partners is necessary. Resupply from the original vendors of the same compounds can become difficult if the compound is older than three years. If this happens during the course of a project, active molecules have to be re-synthesized either in-house or by a third party, which may create additional costs or restrictions for project timelines. The exact replacement of lost compounds, however, is not a stringent prerequisite for our general concept of a primary screening library. We can, in many instances, substitute lost chemical space with similar structures. This approach benefits from the market-driven portfolio change of commercial vendors and their increasing chemical variety. Newly gained knowledge concerning favorable compound properties is applied continuously to compound acquisition. Taking all these considerations into account, a screening library can still be built and maintained relying on commercial vendors only.

Library attrition is also caused by decomposition and precipitation of screening compounds. Dimethyl sulfoxide (DMSO) is the solvent of choice for handling and storing compound stock solutions. But storage in DMSO at $-40\,^{\circ}C$ to $-20\,^{\circ}C$ can cause a significant decrease in chemical quality [49]. Decomposition is most likely the result of multiple chemical reactions that are not fully understood. In many instances, hydrolysis and oxidation by DMSO or oxygen are responsible. Precipitation is expected when the concentration of water within the DMSO stock solution increases beyond 5%. Water and oxygen are predominantly incorporated through air and humidity whilst handling the compound solutions. These factors are not completely avoidable when performing a flexible sample preparation, for example, dissolving and distributing substances in different plate formats. Therefore, we have to accept a limited frequency of degradation and precipitation. Nevertheless, we try to preserve the quality by monitoring the chemical integrity of the stored samples, using

enhanced automated analysis by high-throughput liquid chromatography combined with mass spectroscopy (HPLC-MS) and nuclear magnetic resonance (NMR). Compound quality assessment is used to control and improve compound acquisition, storage, and handling methods, as well as for selection of compounds during the hit-to-lead process.

Compound Handling and Supply Logistics

Compound Logistics provides quick and reliable services such as delivery of compound samples in the most convenient format for the user while preserving the integrity and quality of the library [50]. The ideal way to achieve both objectives would be to store and handle solid compounds and to prepare and deliver samples for screening on demand. This would prohibit the decomposition and precipitation that frequently occur with liquid handling procedures. However, solid sample handling involves laborious manual operations or complicated automated systems for storage, weighing, and dissolving. This, in turn, requires a tremendous investment in laboratory automation and personnel. In the context of a small research unit, we therefore prefer storage and handling of liquid samples because sample formats and amounts can be manipulated with greater ease and flexibility, and processes can be automated at lower costs. But frequent dilution and transfer steps entail negative impacts on library and sample quality. In order to minimize the negative impact of air oxygen and humidity, we use the inert gas argon and dry air conditions for the preparation of DMSO stock solutions. Further damage and material losses are avoided by minimizing the number of liquid handling steps. Compound and sample logistic processes are continuously monitored for potential improvements. The desired reduction of amounts and processing steps is greatly favored by novel equipment that allows more accurate and more miniaturized pipetting and more flexible handling of different formats.

Intellectual Property

Acquisitions from commercial vendors always bear the risk of intellectual property loss to competitors. This issue is observed when competing pharmaceutical research units use the same targets for screening (Komesli, Organon, Newhouse, personal communication). Its impact on animal health lead discovery is less clear. Exclusively synthesized libraries provide novelty of structures and a synthesis route enabling rapid exploration of a defined chemical space. Both factors are favorable for patent protection of lead compounds. However, such exclusive libraries are usually less diverse and more expensive. Due to these constraints, our library concept is still based on compound acquisition from commercial vendors. Nevertheless, by being an animal health division within a larger pharmaceutical company, our discovery efforts can profit from a wealth of libraries exclusively synthesized for other indications within the company, such as for human therapeutics.

Organization of Resources

Lead discovery in animal health has to perform the same closely linked tasks as lead discovery in pharmaceutical research for human diseases, that is, compound management, assay development, target production, HTS, and molecular pharmacology. The complexity of the discovery process demands flexibility from people and technical resources in order to manage scientific exploration and routine work using different technologies and different degrees of automation.

Given the relatively small size of our group working in animal health lead discovery, organizational requirements are challenging. Compound Logistics not only has to cover the full supply chain for screening compounds from purchase of the material up to delivery of ready-to-screen samples in different formats, but also has to perform quality analytics and physicochemical assays, including data processing. Target production includes the exploitation of various target proteins from bacterial, ecto-, and endoparasitic sources, which requires diverse technologies for recombinant expression (e.g., *E. coli*, *P. pastoris*) and native preparation of these targets directly from bacteria or parasites (fleas, ticks, nematodes, etc.) to produce the required target proteins in suitable quality and quantity. As nearly all target families known from human pharmaceutical research are also interesting for animal health, diverse assay technologies, such as enzymatic, protein–protein binding, ion channel, microbial, parasite or cellular assays are required for screening. During the hit-to-lead phase and later stages, additional *in vitro*, *ex vivo*, and *in vivo* biological assays are performed in parallel with physicochemical tests for further characterization of compounds [51].

In general, animal health specific cell lines and assay technologies are developed in-house. The existing assay panel is supplemented with internal and external capabilities in instances when the molecular models from human pharmaceutical research can be directly applied to animal health questions, which is often the case with molecular pharmacology, toxicology, and ADME studies. The latter assays are either established in-house or performed in collaboration with external organizations depending on a "cost to benefit" ratio. Regardless, competencies have to be established in-house to interpret the whole set of study results.

Conclusion

Most pharmaceutical research units have a clear organization of people and tasks oriented along the discovery process. Here we show how this well proven organizational model is applied for lead discovery in an animal health research unit. Precisely defined objectives and workflows are needed as a basis for communication and action. Common learning and sharing of experiences without borders is crucial for project success. In the past seven years our research experienced a broadening of scopes, a better translation of product requirements into objectives, and a constant re-evaluation of feasibilities in the context of a changing technology environment.

Regarding this process of striving for improvement and innovation we can summarize the progress made up to date: our efforts have begun to pay off by filling the research pipeline at an increasing pace. The future will show how this fact will be reflected in successful products on the market.

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8
Approaches Towards Antiparasitic Drug Candidates for Veterinary Use

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Abstract

This chapter reviews the approaches towards the discovery of new antiparasitic drug candidates for veterinary use. A retrospective analysis first summarizes the historical sources of veterinary antiparasitic drugs and the partnerships of animal health companies with other industries for the exchange of drug candidates. The second part focuses on an introduction of the current approaches used for the identification of antiparasitic leads. Following the order of increasing biological complexity, a detailed analysis of the advantages and limitations of each approach is presented. Finally, the chapter concludes with a description of the different workflows implemented for optimizing the various types of leads into antiparasitic drug candidates.

Introduction

Infectious diseases are caused by a variety of organisms (e.g., viruses, bacteria, parasites, fungi) which affect both humans and animals. In the medical field the term "parasite" encompasses protozoa, helminths, and arthropods that either provoke diseases in their own right or, in the case of arthropods, are important vectors of other pathogenic agents (www.medterms.com, http://www.cdc.gov/ncidod/dpd/aboutparasites.htm). The parasites are sometimes also categorized as endoparasites or ectoparasites, depending respectively whether they live inside or outside the body of their host [1]. For brevity, this chapter focuses on metazoan parasites. The combat against endo- and ectoparasites is not only essential to animal welfare but is also playing an essential role in the human food safety chain and is of fundamental importance for human health. As an illustration, public–private partnerships have been initiated to support antiparasitic drug discovery programs to respond to the acute need for efficient antiparasitic drugs for treating populations in developing countries [2] (see also Chapter 3).

In light of this development, the expertise of veterinary pharmaceutical companies in finding and optimizing antiparasitic leads becomes even more valuable; and the sharing of this expertise should be further encouraged [3]. From a general point of view, the tools and processes involved in the progression of a veterinary candidate from hit to drug compares well to the human pharmaceutical landscape. The principal difference probably resides in the higher fragmentation of the animal health market, characterized by a high diversity of hosts and of parasites which makes broad-spectrum drug candidates the favorite outcomes of veterinary drug discovery programs to ensure a return on investment.

The following sections first review the historical strategies used to discover anti-infectious/parasitic drugs and pathways between animal health and other industries. Then, three principal approaches constituting the basis of most drug discovery processes towards antiparasitics are presented; and finally the principles guiding the conception of lead optimization workflows are detailed.

Historical Strategies Towards Veterinary Antiparasitic Drugs

By looking back at the strategies used for the discovery of veterinary antiparasitic drugs, two dominant approaches can be identified. The first is chance finding (part of it serendipity) and the second is structured research [4, 5]. Serendipity, the discovery by accident and sagacity while pursuing another goal, and chance have been important sources of some major drugs for treating infectious diseases of both humans and animals [6]. Piperazine, tetramisole, and praziquantel (Figure 8.1) are examples of compounds for which chance finding played a pivotal role in generating the drug candidate, since either the structure or the activity were fortunate discoveries. The discovery of the anthelmintic properties of piperazine can be traced back to the accidental observation by the French pharmacist Boismare that patients were cured from *Ascaris* infection while being treated against gout [7]. Veterinary domestic

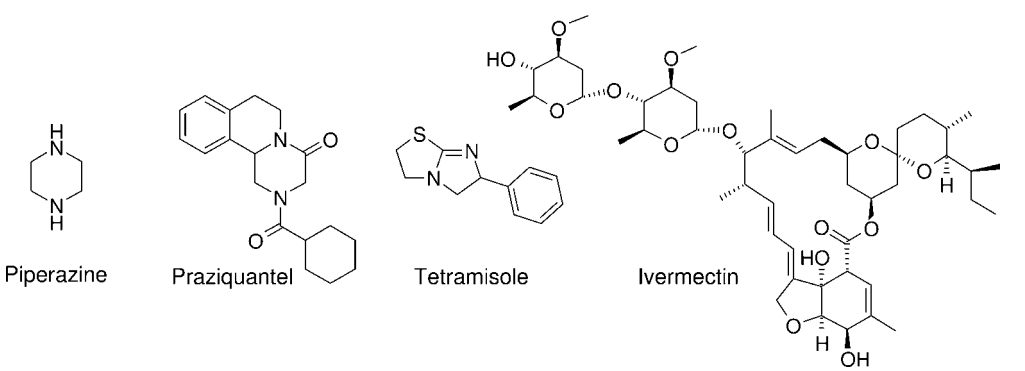

Figure 8.1 Exemplary antiparasitic drugs.

and companion animal applications then trailed behind successful anthelmintic use in man [8, 9].

Interestingly, the first report on the anthelmintic activity of praziquantel also suggests that the discovery of activity against schistosomes and cestodes was a fortunate coincidence [10]. Pyrazinoisoquinolines were originally synthesized at the E. Merck company for an intended use as tranquilizers [11]. After the compounds failed to fulfill the intended profile, they were passed to Bayer AG for veterinary screening; and, out of about 400 compounds, praziquantel was further developed for anthelmintic use. This case shows that chance finding and structured research do not compete against each other but are rather complementary. As another example, tetramisole originated also from a structured anthelminthic screening. However, it was only by comparing the metabolites derived from a compound that showed activity in one species but not in the other that the active compound was identified. In that way this discovery was also a consequence of the fortunate choice of these two models and thus of chance [12]. In contrast, structured research is an approach directed towards the discovery of drugs possessing specific profiles and addressing defined therapeutic uses. In the 1960s, animal health companies started and then gradually increased their investments in structured antiparasitic research, which resulted in the introduction of a variety of novel antiparasitics onto the market [13]. Among these success stories, the discovery and development of ivermectin (Figure 8.1) might be regarded as the climax of such initiatives [14]. In the early 1980s, mechanism-based drug generation was broadly adopted by the human pharmaceutical industry and became the predominant structured approach [15]. However, it took about 15 years more until the promise of the target-based approach, and in a broader sense the rational design of veterinary antiparasitic drugs, was fully recognized [16–20].

At the same time the challenges to optimize broad-spectrum antiparasitic drugs by using *in vitro* tests were illustrated by the example of the avermectins [21]. It was realized that, given the high diversity of parasitic species and their target hosts, the *in vitro* optimization of potency against one parasite has a high probability of resulting in a sub-optimal drug for treating other species. In addition, the advantages of the mechanism-based approach (such as control of target-related toxicity) could easily be offset by loss of the natural context. Recently, Adam argued that the optimal drug discovery concept may reside in an optimal combination of the strengths of chance-based screening methods with the advantages of the target-based approach and thus allow the development of therapeutically active compounds, making use of well understood modes of drug action [22].

Interactions between Veterinary Drug Discovery and Other Industries

The similarities in human and veterinary drugs requirements have opened the way to the exchange of drug candidates and even drugs between both parties. Thus, several

approved animal drugs contain the same pharmaceutical active ingredients as human drugs [23], however, in some cases for a different indication. As an illustration, the demand for new pet drugs recently benefited from the transfer of human drugs [24]. Yet, it is not a one-way street and, particularly for tropical diseases, human drugs often originate from the veterinary pharmacopoeia [25]. For example tropical diseases such as Chagas' disease, schistosomiasis, and onchocerciasis are respectively treated with nifurtimox, praziquantel and ivermectin, which were originally developed for veterinary uses; and the urgent need for new treatments of human parasitic infections emphasized in a recent issue of *Expert Opinion on Drug Discovery* attest to the relevance of this subject [26].

As part of this publication, an article [27] disclosed the target profile of a novel human trematocidal compound which is comparable to the characteristics of a modern veterinary anthelmintic (Figure 8.2). Similar drug criteria are also posted by The Institute for One World Health, (http://www.oneworldhealth.org/business/opportunities.php) an organization that identifies new medicines for infectious diseases affecting developing countries and develops them by partnering and collaborating with pharmaceutical industries and non-profit organizations. In addition veterinary drugs might also be of value in more distant human indications. As an example, halofuginone, a veterinary anticoccidial drug inhibiting angiogenenesis is currently in clinical trials for the treatment of advanced solid tumors and HIV-related Kaposi's sarcoma (http://www.clinicaltrials.gov) [28].

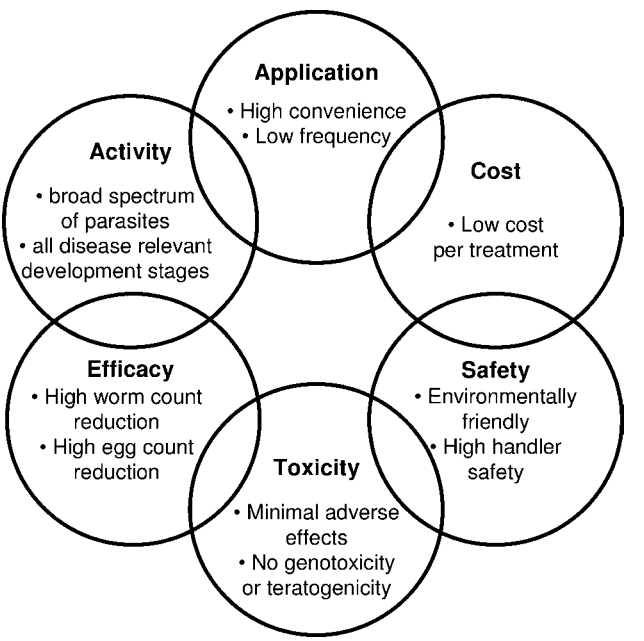

Figure 8.2 Target profile shared by a human trematocidal and a veterinary anthelmintic drug inspired from Keiser and Utzinger [27].

In addition to its partnership with human pharmaceutical companies, the animal health industry has also a privileged relationship with agro companies in the ectoparasitic field. In a large majority of cases, the repositioning process is quite straightforward and, as a consequence, only a few examples of lead optimizations leading to veterinary ectoparasiticides have been published in the literature. First, there is a profiling phase, aimed at determining the potential of pesticides as veterinary drugs; then the original crop protective application is extended to a veterinary use, principally by the development of a topical formulation (e.g., spot on, pour on). Nonetheless, the medicinal chemistry efforts on the nodulisporic acid that aimed at a new oral flea and tick drug is an inspiring example of a successful optimization towards a veterinary oral ectoparasiticide [29]. In the end, it could be envisaged that the pharmacokinetic and pharmacodynamic knowledge generated during the repositioning of agro compounds for a veterinary use might be in turn a good entry point for human drug discovery programs directed for example against head lice infestations. This would open an opportunity to finally find efficacious successors to the currently prescribed old ingredients and thus address the concerns of developing resistance [30].

Current Approaches Towards Veterinary Antiparasitic Drug Candidates

It is largely accepted that the lengthiest part of the drug discovery investigations is dedicated to the multi-objective optimization of lead compounds into drug candidates. The profile of such candidates is generally characterized by conflicting requirements such as high efficacy and low toxicity and might be guided by the principles of the Pareto optimization. The Pareto Optimum was originally developed for a use in finances, but it can also be applied to modern drug discovery [31]. The goal is first to define a minimum set of parameters (e.g., half maximal inhibitory concentration, minimum effective concentration, aqueous solubility) necessary for qualifying a drug candidate and then to determine simultaneously the values of all these parameters. The optimization process is ultimately the result of numerous tradeoffs between conflicting attributes embodied in one compound in order to find the therapeutically optimal solution. The optimization process of an antiparasitic lead into a drug candidate may be split into three principal phases (Figure 8.3). During the first phase (Q1–Q2), *in vitro* assays are used as optimization tools, allowing the screening of thousands of compounds per year. This first multi-dimensional optimization typically focuses on finding the best balance between activity on the target or on whole parasites (e.g., bioscreen), the *in vitro* toxicity (e.g., cytotoxicity, genotoxicity), and the physicochemical properties (e.g., stability, solubility). This optimization is generally supported by *in silico* tools such as molecular modelling and docking, pharmacophore design, virtual screening,

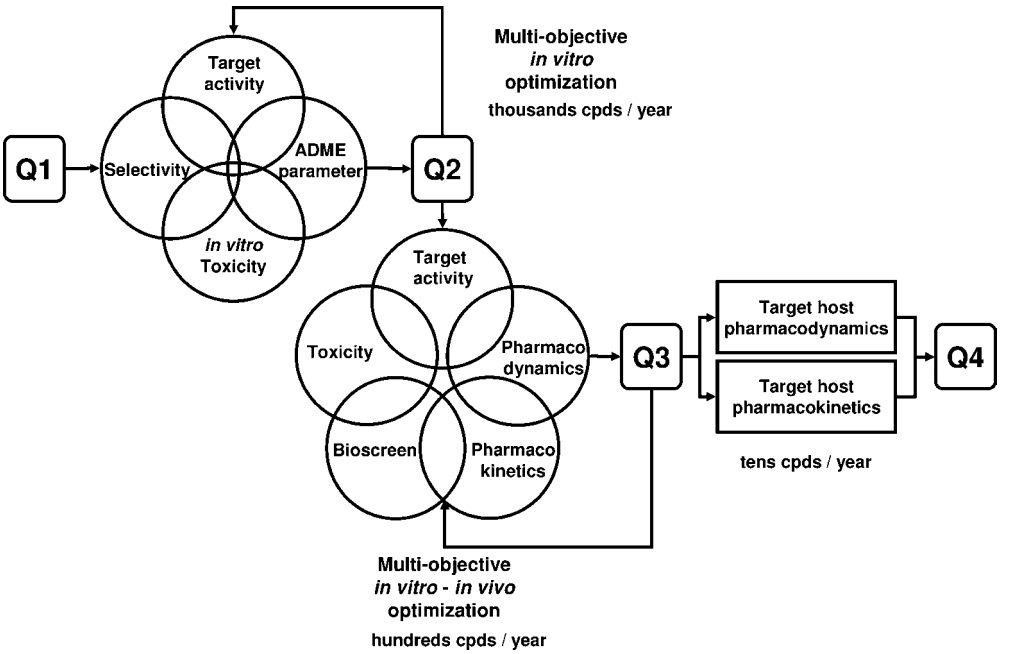

Figure 8.3 Multi-objective lead optimization process. Quality level 1 (Q1): lead status; quality level 2 (Q2): *in vitro* lead; quality level 3 (Q3): *in vivo* lead; quality level 4 (Q4): drug candidate.

and data-mining tools. The second phase (Q2–Q3) additionally includes animal models to investigate the translation of the *in vitro* profile to *in vivo* conditions. This phase should enable the optimization towards compounds that fulfill all prerequisites for entering a validation study in target species. Such studies are then performed as the final stage of the optimization process (Q3–Q4). During this phase, the pharmacokinetic, pharmacodynamic, and toxicity profiles of the selected compounds are determined in at least one target host against one target parasite and enable the selection of the most promising candidates that will enter a predevelopment phase.

Due to the low throughput of these studies and the multi-causal nature of the phenotypes observed, new optimization directives cannot easily be drawn from the experimental results. In that sense, this final stage of the optimization has more the character of a profiling than an actual optimization. Compared to the optimization sequence usually implemented in the human pharmaceutical industry, less pronounced translational gaps are present between the target screens, the phenotypic or function-based screens and animal models. In addition, the optimization of veterinary leads is concluded by a first proof-of-concept study in one target species. Finally, in cases where the information content regarding either the validation, or the druggability of a novel target or the relevance of a phenotype for a given disease is limited, *in vivo* pharmacodynamic studies can be performed in parallel, in model and

in target animals, provided that toxicity issues have been adequately addressed. Interestingly, although one could expect that a great variety of approaches would have to be implemented to address the specificities of multiple antiparasitic indications, it seems that only three are able to cover all processes in place. The distinction of these approaches has been made on the basis of the biological complexity level of the test systems and resulted in the following nomenclature: mechanism-, function-, and physiology-based approaches [32, 33]. In the following sections, the particularities of each approach are presented and comparisons are made between the human and veterinary fields.

Mechanism-Based Approach

In recent decades, the mechanism-based or target-based/centric approach has attracted most of the attention of pharmaceutical companies. The principal argument in favor of this approach is the postulate that leads (later, drug candidates) derived from a mechanism-based approach elicit their pharmacological effects by selectively acting through defined mode of actions, therefore preventing side-effects that result from undesired off-target interactions. From a more technical point of view, the implementation of this approach has also been facilitated by the combination of concomitant progress made in various disciplines. The sequencing of model organisms of infectious species delivered a great number of target gene candidates, thus building the foundation of the mechanistic approach. In addition, the development and generalization of highly automated miniaturized assays dramatically increased the average screening throughput which, combined with the advent of the combinatorial and parallel chemistry methods, allowed the screening of thousands of compounds daily. However, as it is often the case when a new concept is introduced and is rapidly gaining prevalence in a field, the limitations associated with applying the mechanistic approach to the veterinary field have been underestimated [34]. The first of them is encountered at an early stage of the drug discovery process, when the validation of the target is conditioning the initiation of further activities [35]. Following the annotation of a gene of interest in model organisms such as *Caenorhabditis elegans* or *Drosophila melanogaster* and the demonstration of the presence of homologs in a broad range of target parasites by means of comparative genomic tools, the validation of the target in a disease-relevant parasitic species is a key issue. In most cases, since the validation by functional genomics remains extremely challenging for most disease-relevant parasitic species, this process still relies on a chemical validation achieved by the testing of a known modulator of the targeted receptor [13]. As a consequence, the opportunities to validate innovative targets are rather limited. In addition, the parasite's morphology often varies dramatically along the various development stages composing its life cycle. Thus, important changes occur in the up- and down-regulation of protein expression, which might have a decisive influence on the therapeutic relevance of targeted receptors. Further limitations are also encountered at the high-throughput screening stage. Under most screening conditions,

the target and the compounds are confronted in a significantly more simple way when compared to the physiological conditions the future drug will encounter [36]. Therefore, during the hit-to-lead phase, the potential of the hits may be either over- or underestimated, thus resulting in the selection of sub-optimal leads (see also Chapter 19).

As a result, leads issued from a mechanistically driven selection offer the advantage to be clearly associated with a distinct and chemically validated mode of action. Depending on the level of information regarding the binding mode of the compounds, this may constitute a decisive asset for a rapid progression in the optimization phase, which will also be further supported by the high throughput of the screening tools. However, in the case of novel targets, demonstration of the transferability of the *in vitro* activity to the *in vivo* conditions and the therapeutic relevance of the target in all disease-relevant parasitic stages are often uncertainties that need to be addressed during the optimization phase.

Function-Based Approach

Function-based screening and in a broader sense biology-driven drug discovery recently benefitted from a reawakening of interest as it became evident that the expectations placed in a purely target-based approach were not all met [37]. It was realized that screening individual isolated targets may have been an over-simplification of the disease models, which is not only unable to reliably mimic complete biological pathways, but also per essence does not reduce the risk of unanticipated off-target interactions. In order to address these issues, a step back to the function-based approach was made, involving a higher degree of biological complexity. In this case, the restoration or the interruption of a disease-relevant function involving the concerted action of a group of individual mechanisms is monitored. Moreover, since function-based screens include part of the biological barriers the compounds will have to cross in order to elicit their pharmacological effects, they also constitute helpful tools for getting the first insights on the optimal set of physicochemical properties. In contrast to the mechanistic approach where a common definition would apply to both the human and the veterinary fields, the understanding of the function-based approach in the veterinary antiparasitic field might sensibly differ from the human health perspective. In the human pharmaceutical context, a large majority of the function-based screens are based on whole-cell assays [38], as is the case for GPCRs [39]. In the veterinary antiparasitic field, however, functional screens also called bioscreens involve a higher degree of biological complexity since it is based on the screening of whole organisms, such as helminths, arachnids, or insects. Under this consideration, this type of assay is at the frontier of the physiological approach, since the functional parameters observed are in direct line with the symptoms of the disease. Thus compounds selected on the basis of bioscreens results possess not only the required intrinsic activity but also the desired physicochemical properties to be efficiently absorbed by the parasitic species. In addition by taking advantage of the substantial amount of information gathered over the years on

the association of distinct phenotypes to various types of targets, it is possible to propose a tentative mode of action and then validate this hypothesis on the target level. As in the case of the mechanistic approach, the function-based approach also has its own limitations [40]. Particularly in the case of the endoparasites, finding artificial conditions that maintain the parasite for long enough in the absence of their natural hosts is a critical prerequisite for implementing such assays, which constitutes a great challenge and often a strong limitation. In addition, it can be reasonably postulated that the morphological changes mentioned earlier will also influence the transfer of compounds into the parasites. For these reasons it would be of great interest, at least for the most prevalent parasitic species, to have all disease-relevant stages available for screening. Unfortunately, this challenge remains until now unmet. Finally, although some automation has been introduced in this field [41], the screening throughput of functional assays is extremely limited when compared to target-based screens.

Accordingly, a lead selection on a functional basis results in compounds possessing a demonstrated *in vivo* biological activity against relevant parasitic species. However, as the range of assays fulfilling the throughput requirements for lead finding and optimization programs remains limited, activity is not necessary determined on the most relevant species or development stages [42] and thus requires confirmation in animal trials. Finally, although some information can be captured from the observed phenotypes, elucidation of the governing mode of action of bioactive compounds is usually a very challenging task, as the biological activity may result from the concerted action of various mechanisms [43].

Physiology-Based Approach

The highest degree of biological complexity is reached with the physiology-based approach. This approach differs from the previous two in the sense that, for obvious animal welfare reasons, it is not intended to be used for the screening of large collection of compounds, but rather for selecting candidates from a set of compounds possessing promising *in vitro* profiles. As was already the case for the function-based approach, scientists involved in human and veterinary health may have different perceptions of this notion, depending on their therapeutic area of interest. In the human health context, the physiology approach has been defined as the investigation of the effects of compounds on the disease symptomatology. This type of experiment is usually performed either in isolated organs or in model animals. In the veterinary field, however, physiology-based screens aiming at the selection of ectoparasiticides can be advantageously performed directly in target animals. In this field, identical parasitic species can be tested in functional and physiological assays, thus making translation of the activity predominantly dependant on the physiology of the host and therefore making the use of model animals inopportune. Under this consideration, the physiological screens might thus better correspond to the early clinical trials performed in patients in the human health. However, the veterinary anthelmintic

field compares well with the human pharma situation. In this case, models of the diseases are used and compounds are selected according to their ability to cure laboratory animals (typically rodents) that are artificially infected with target disease-relevant parasites (livestock or companion animal parasites). This type of model offers the advantages of significantly minimizing the amount of compound and parasitic material needed and also reducing the prepatency time required when compared to studies performed in large animals. In addition, apart from their primary selection purpose, studies performed using such models deliver the first orientating information on the therapeutic window of the compounds and therefore are very valuable for determining the initial dose for conducting the first target animal studies. However, because most parasitic species do not undergo a normal development, as they would normally do in their natural hosts, such assays are usually limited to screening against larval or pre-adult stages of parasites. Finally, since transcuticular diffusion was reported to constitute the preponderant mode of drug uptake by endoparasites [44], variations in the gastrointestinal tract physiology between rodents and target animal species might result in a misleading (de)selection of compounds. Therefore, physiology-based investigations aim at validating working assumptions made at earlier phases of the optimization process rather than at screening compounds for initiating new projects. Depending on the therapeutic area of interest, physiology-based experiments thus serve as a proof of principle either for progressing optimized leads into the pre-clinical phase (as may be the case for ectoparasiticides) or (in the case of anthelmintics) for the *in vivo* validation of an *in vitro* activity (mechanistic or/and functional) conditioning the initiation of target animal studies.

Lead Optimization Workflows Tailored to Leads Profiles

In the veterinary field, mechanism- and function-based screenings currently represent the two main sources of leads for starting optimization programs. The following sections present the parameters (according to the origins of the lead) influencing the conception of such workflows in the context of the optimization of lead compounds to veterinary drug candidates.

Leads Derived from a Mechanism-Based Selection

Influence of the presence of a homolog gene in the target species

In the case of leads issued from a mechanism-based selection, a first distinction is made depending on the presence of a homolog gene in the target species. In cases where there is evidence for the presence of the target in the host species, target-associated toxicity constitutes a potential issue. In order to estimate and

monitor this risk, resources needs to be allocated to the implementation of counter screens which will be used as de-selection tools during the optimization. At this stage, it is critical to establish a counter screen not only for each target species but also for all model animals that will be part of the optimization cascade. Equally important is the definition of the de-selection criteria. For this purpose, activity levels of known standards interacting with the target may constitute good starting points. In cases where no benchmark knowledge is available, the expected therapeutic window can be a helpful parameter for setting orientation values. However, as the pharmacokinetic profile might be as relevant as the activity on the isolated receptor, this provisory definition should be challenged during the course of the project and revised in accordance with the results of the first *in vivo* toxicity studies.

Influence of target novelty

A second determinant factor strongly influencing the design of the lead optimization workflow is target novelty. It is commonly accepted that the introduction of a new drug making use of a novel mode of action will be followed after a varying period of time by the emergence of resistant species (see also Chapter 2). This principle also applies to the veterinary field and particularly to the field of anthelmintics where resistance was systematically reported within five to ten years following the introduction of each new class of compounds. Since the past two decades have not witnessed any introduction of new chemotypes, resistance has now been documented against almost every marketed veterinary antiparasitic drug [45]. For this reason, when a new lead optimization project is initiated on a lead interacting with a known target, it is essential to include the resistant receptor in the optimization cascade, or at least to have the resistant parasitic species available for functional screening. If a long-term commitment to the target class is decided, activities might be considered that aim at a better understanding of the mechanisms involved in the resistance against known drugs. In addition, comprehension of the binding mode of the newly developed compounds via docking and modelling studies should be considered as potentially helpful tools for building a working hypothesis and for guiding the optimization strategy.

In the case of lead optimization projects based on novel modes of action, target-related resistance issues are not relevant (however, some attention should still be paid to compound-related resistance and thus to the analogy of the new compounds to known drugs) but other types of uncertainty need to be addressed by the workflow. Among these, the transfer and correlation of the *in vitro* activity to *in vivo* conditions need to be demonstrated as early as possible. To this end, the investigation of compounds in a functional screen should be incorporated in the workflow as soon as a diverse set of *in vitro* active derivatives is made available. Furthermore, as negative results may also be attributed to a sub-optimal uptake of the compounds by the parasitic species, studies enabling clarification of this eventuality should also be considered. For example, this might be realized by applying the compounds in the parasitic species via injection or by investigating the concentration of each compound in the parasites.

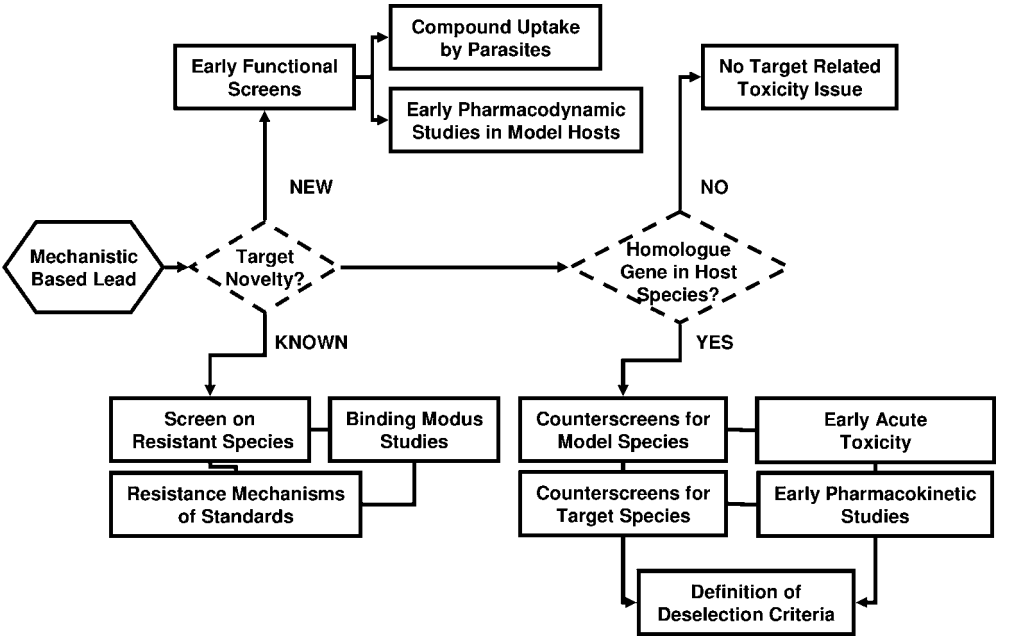

Figure 8.4 Identification of priorities to be addressed by the lead optimization workflow of mechanism-based leads.

Once it has been shown that the modulation of the target is leading to a biological effect in living species, the druggability of the novel ligand needs to be unveiled. A ligand will be declared druggable if it is able to produce the expected therapeutic effect while keeping the side-effects to an acceptable level (Figure 8.4).

Leads Issued from a Function-Based Selection

In the case of projects based on leads derived from a function-based selection and making use of functional screens as an optimization tool, a large part of the work can be covered by a generic workflow. The following paragraph details the generic part and then comments on variations, depending on the therapeutic field of interest. One of the drawbacks of utilizing a function-based approach for the optimization of anti-infective agents resides in its propensity to primarily select biocide compounds (uncouplers, ionophores, etc.). As a consequence, the workflows need to include some tools enabling the discrimination of compounds with a broad toxicity spectrum from compounds possessing the desired antiparasitic properties. But, it should also be kept in mind that negative results have to be carefully interpreted, as they may have different causal sources. In some cases they may relate to a lack of intrinsic efficacy, whereas in others they may be connected to sub-optimal ADME properties. In order to address this issue, chemoinformatic tools (neural networks, principal component analysis) can be efficiently combined to data mining tools (multi-dimensional

analysis programs) to determine which combination of physicochemical parameters characterizes functionally active compounds and, as a consequence, to distinguish poor uptake from poor activity. However, besides the difficulty in interpreting bioscreen results in terms of a structure–activity relationship, the major drawback in using functional screens as optimizing tools remains the absence of information regarding the mode of action. This may not only have direct consequences on the screening throughput and thus on the optimization cycle times, but may also jeopardize the definition of a clear structure–activity relationship as compounds might jump from one target to another along the optimization process. It is thus essential, in parallel to the optimization work, to allocate some efforts towards gaining knowledge (as much and as fast as possible) on the putative mode of action [46, 47]. Unfortunately, the veterinary antiparasitics field still lacks efficient tools to address this issue and investigations mostly rely on the critical interpretation of the phenotypes and on secondary screening against species resistant to known drugs.

Specificities of ectoparasitic functional leads

Depending on the therapeutic area of interest, some specificities might be incorporated in function-based workflows. For example in the ectoparasitic field, the phenotype and the efficacy are parameters of equal importance. Thus, the delay between the contact of the parasite with the compound and the observation of the effect should lie in a defined time-window. An excessive time needed to neutralize the parasite may enable the transmission of diseases following a tick bite, whereas too fast a kill may result in a long-term attachment of the parasite to its host. In order to get clarity on such issues, which are in part dependant on the physiology of the host, the workflow should include a proof-of-principle study as soon as a compound possessing a sufficient level of efficacy is identified. As ectoparasiticidal compounds are also preferably applied topically (as pour-on or spot-on formulations), physicochemical parameters such as aqueous solubility, volatility, or stability to light exposure are of particular importance and will be integrated for the design of new derivatives.

Specificities of endoparasitic functional leads

In the case of the optimization of anthelmintic leads, most functional assays are based on larval stages of the parasites that only cover part of the development cycle taking place inside the host. Moreover, unlike in the ectoparasitic field where the notion of functional activity is mostly a synonym of a kill effect, anthelmintic bioscreen results are expressed as the capacity of compounds to induce a broader variety of phenotypes, such as paralysis or reduction of motility or, on the contrary, hyperactivity. In order to ensure that compounds being optimized have the potential to eradicate the disease-relevant development stages, and to ensure that the phenotypes observed in functional assays will lead to an effective reduction of the parasitic infection, *in vivo* studies against target parasites in model species must be conducted as soon as compounds possessing a promising *in vitro* profile are identified. Alongside these pharmacodynamic studies, it is also of critical importance to assess the pharmacokinetic profile of the compounds in order to estimate their potential to

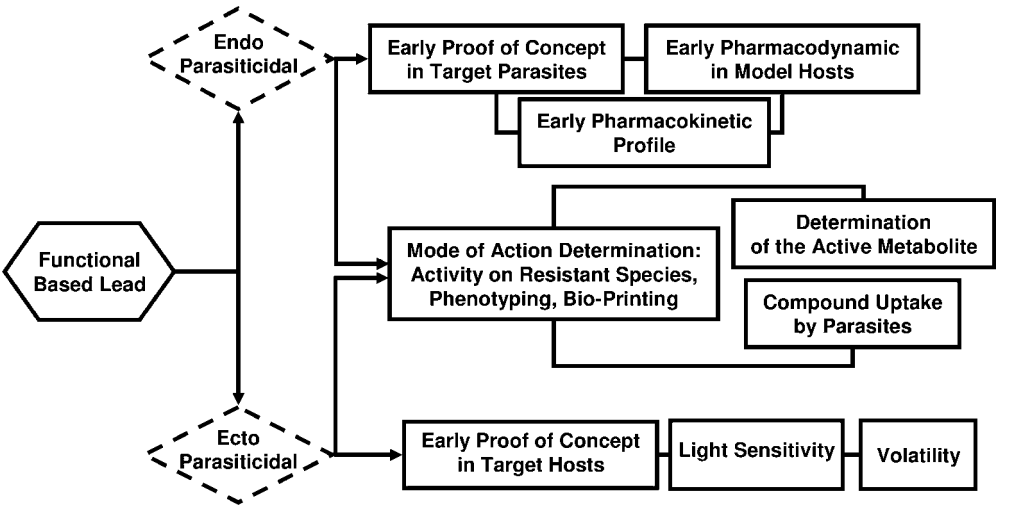

Figure 8.5 Identification of priorities to be addressed by the lead optimization workflow of function-based leads.

reach parasitic species or stages that may be found in a variety of organs (lungs, liver, heart, etc.; Figure 8.5).

Other Factors Influencing Lead Optimization Workflows

Besides the nature of the therapeutic field of interest and the approach followed for generating the lead, the chemical structure of the lead also dramatically influences the priorities to be addressed during the lead optimization phase. This would for example be the case of an optimization project based on a functional approach and on a lead whose structure suggests that the compound might not act as itself but only after metabolic activation or chemical transformation [48]. Leaving room for uncertainty regarding this matter not only jeopardizes the optimization of the lead into a drug candidate, but it also negatively impacts the intellectual property of the newly developed drug. Prioritization of structural changes within the lead structure is also aligned to the target drug profile. For example, it might be advisable to study the structure–taste relationship when targeting an oral application with enhanced palatability. Similarly, when targeting a topical application in companion animals, structural modifications leading to compounds having a neutral coloration are favored. Other structure-related parameters (such as novelty) also influence the optimization of a lead. Beside the obvious intellectual property aspects, the novelty (or more precisely the analogy of the lead to other drugs) has a determinant impact on its capacity to break resistance [49]. Thus even in the case of a lead making use of a novel mode of action, sufficient attention should be allocated to this parameter during the optimization process. On a general point of view, all attributes that relate to the structure, such as aqueous solubility, stability, lipophilicity, cytotoxicity, genotoxicity,

synthetic accessibility, cost of synthesis, and patentability might be integrated as a generic part of the lead optimization workflow.

Conclusion

To conclude, the tools and processes involved in the discovery of veterinary drugs compare well with those used in the human pharmaceutical industry. Whereas the lead optimization workflows implemented in the veterinary field take advantage of the easy access to functional and physiological assays and also the opportunity to perform proof-of-concept studies early during the drug discovery process, the discovery and optimization of lead compounds for an application to humans can count on more advanced tools for the validation of the targets and on a deeper understanding of the influence of pharmacokinetic profile on pharmacodynamic properties. In both fields, however, the objective of these processes is to deliver new drugs which satisfy efficacy and safety requirements and can be manufactured via consistent and accurate processes [50]. It is therefore likely that insights in the veterinary lead optimization process and its results will be helpful to human pharmaceutical research, and vice versa. Fostering mutual understanding and cooperation between veterinary and human drug discoveries will be advantageous for both disciplines, and also for public–private partnerships in their efforts to find new drugs for the treatment of neglected diseases.

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9
Learning to Relate Structural Space to Property Space

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Abstract

Drug discovery aims at finding a previously unknown applicability of a substance as a drug. It entails the mapping of properties of substances against a set of criteria which define the applicability. It would be preferable to start from desired properties, as given by the criteria, and deduce *de novo* the substances which would have these properties. This would allow the design of the most suitable chemical out of the virtually infinite number of chemicals structurally conceivable. The knowledge base, however, by and large does not exist. Therefore, the starting points for drug discovery are not the desired properties, but physically existing chemicals which are then exposed to suitable model experiments in high numbers to determine their properties. We were interested in the question of whether relationships could be obtained from the calculated properties of registered veterinary pharmaceuticals which could guide the selection of compounds for property determination. We learned that calculated properties mainly relate to the structural origin of the active pharmaceutical ingredients, either natural or non-natural. In contrast, measured permeability and lipophilicity values allow for differentiation between classes, such as those having different application forms, but they are independent of the structural origin of the active pharmaceutical ingredients. Translation between these simple structural descriptors and property space does not seem feasible. We encourage the continued search for descriptors which allow a correlation of the structural space with the property space. Veterinary drug discovery, and pharmaceutical research in general, will greatly profit from the resolution of this apparent dichotomy.

Why Making A New Drug Fundamentally Differs from Making A New Car

Drug discovery aims at finding a previously unknown applicability of a substance as a drug. This holds true in both the academic and the industrial environment, and for both the human pharmaceutical area and animal health. The chemical structure of the substance might be known or unknown at the outset. Drug discovery thus entails the mapping of properties of substances against a set of criteria which define their applicability. It would be preferable to start from desired properties, as given by the criteria, and deduce *de novo* the substances which would have these properties. This would allow the design of the most suitable chemical out of the virtually infinite number of chemicals structurally conceivable. Typically, classic engineering industries like the car industry use this approach. Starting from the desired attributes for a new car model, engineers can design the new model and rapidly move into prototyping and production. This sequence is enabled by a broad and well established knowledge base concerning which types of materials and constructions have to be used to yield certain attributes of the finished car. In the drug discovery world, the situation is reversed, because this knowledge base is considerably weaker. Typically, the starting points for drug discovery are not the desired properties, but physically existing chemicals which are then exposed to suitable model experiments in high numbers to determine their properties. The physically existing substances, and in particular the limited substances available to an individual drug discovery unit, constitute only a minute fraction of the chemicals that are structurally conceivable. Consequently, the probability that the measured properties of a compound will in fact meet all the desired and predefined applicability criteria is rather low. Therefore, a drug discovery process must include a process of iteratively changing the structure of each chemical by chemical synthesis and then determining and rating the properties [1]. Coming back to the comparison with the engineering industries: a car manufacturer in contrast would never generate a huge number of car prototypes and test them for the desired attributes afterwards in iterative cycles, as a drug discovery unit does with compounds and test models.

How to Address this Difference

In the 1990s, industrial drug discovery strived to decrease costs per determination step by the introduction of high-throughput methods for synthesis and property determination. After the initial euphoria regarding the apparently limitless possibilities of these high-throughput methods, the use of very large sets of compounds in the screens proved to be impractical and, further, added to the costs [2]. In

particular, the follow-up, confirmation, evaluation, and prioritization of the correspondingly large numbers of hit or lead compounds, having at least a few of the desired properties, proved to be costly. In the past few years, we can see a clear trend towards smarter selection of the compounds to be submitted to property determination. The smarter selection of compounds in this regard means a nonrandom, targeted reduction in the number of compounds. This is precisely where drug discovery needs to establish the knowledge base regarding the relationship between structural space and property space, for example, by learning which structural changes will lead to which changes in the compound's properties. The world of structure is the domain of the chemist, whereas in the context of pharmaceutical research, the world of properties is the domain of the biologist. These two scientific disciplines need to bring their different views and knowledge on the same topics into a common knowledge base. Back in 1907, the importance of re-establishing the interaction between these two scientific disciplines was already being stressed by E. Fischer [3], "as the great chemical secrets of life are only to be unveiled by co-operative work." Compound names like fipronil, avermectin or fenbendazole evoke very different associations. For the biologist, they stand for compounds with typical antiparasitic properties. For the chemist, they are typical representatives of three structural classes, characterized by well defined and differentiated constitutional elements. Applying Johannsen's [4] definitions of genotypes and phenotypes, we can state that the compound as characterized by distinct constitutional elements is a molecular genotype for the chemist, while the same compound as characterized by its antiparasitic properties is a phenotype for the biologist [5] (Figure 9.1).

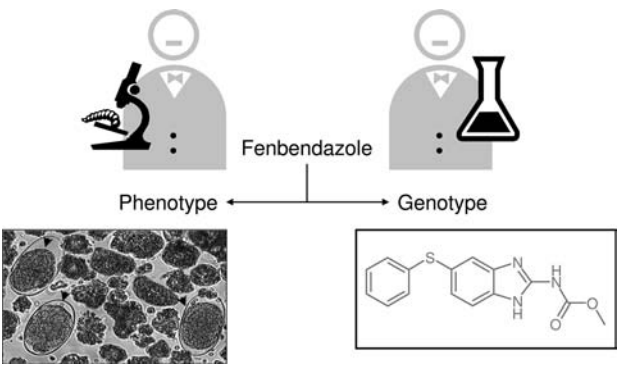

Figure 9.1 A drug-like fenbendazole, as viewed by a biologist (left) and a chemist (right): the ovicidal effect on *O. dentatum* eggs versus the structural formula.

Clarifying the Terms Relating to Experimental and Computational Methods

Property criteria comprise a disease-specific and a nondisease-specific term. The disease-specific term in the context of antiparasitic drug discovery might be the activity of a substance against pathogens like parasites. The nondisease-specific term might be the toxic properties of a substance or its pharmacokinetic behavior in the patient. In recent years, the goal of selecting substances in a nonrandom fashion was approached by focusing on the nondisease-specific properties of the compounds. This approach is based on the assumption that a general principle – drug likeness – would predispose a substance to be either a drug or not a drug. The major contributions to drug likeness are the absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties of a drug. Each property in itself is the sum of a large number of individual properties of a drug molecule. Let us conduct a thought experiment: If one assigned a greater importance to drug likeness than to the disease-specific term, then there should be a drug-likeness screen before the screen for disease-specific activities. In reality, only a few components of drug likeness can be represented in screens, and then with generally only low throughput. A reversal of the current screening paradigm has not happened so far [6].

Drug likeness is fundamentally a function of the structure of the drug [7], as are the disease-specific properties. To compensate for the low throughput, calculations of "molecular," "physical" or "physicochemical" properties were introduced to tackle the term of drug likeness. These terms are used inconsistently in the literature [8–10]. We would like to propose the term "constitutive properties" instead. Lipinksi's rule of five (Ro5) [11], used for the prediction of absorption and permeation properties of potential drug molecules in human pharmaceuticals, can serve to elucidate this unambiguous term. The Ro5 parameters are the number of H-bond donors, the number of H-bond acceptors, and the values of the molecular weight and the calculated logP (clogP) of a molecule. They are not physical or physicochemical properties of the molecule, but rather constitutive properties. They are simplifying abstractions of the complete description of the chemical structure of the molecule, since only by simplification do the common constitutive properties of the drug molecule become recognizable. The determination of constitutive properties requires nothing more than the structural formula and a convention regarding how the structural formula is to be translated into the abstraction, for example, the algorithm used for the calculation of the clogP. These constitutive properties correspond to a molecular genotype. But, the determination of a physical or physicochemical property necessitates substance, as well as a method of measurement for the property of interest, for example, the distribution coefficient between water and 1-octanol. These measurable properties or functions of a molecule correspond to the phenotype. We can thus assign computational ("dry bench") methods to the world of the genotype and experimental ("wet bench") methods to the world of the phenotype.

Definition Box

Structure	Number, type, connectivity and spatial distribution of the atoms in a molecule.
Structural space	Virtual, multidimensional space populated by an ensemble of chemical structures.
Property space	Virtual, multidimensional space populated by an ensemble of compound properties.
Constitution	Number, type, and connectivity of the atoms in a molecule, omitting information on the spatial distribution. Single atoms or groups of atoms form constitutional elements.
Constitutive properties	Properties which result from calculations based on the descriptors of a molecule's constitution.
Genotype	Representative of a particular gene, irrespective of the nature of the gene. In the case of a molecular genotype, this refers to the chemical structure of a molecule.
Phenotype	Representative of any apparent and thus observable characteristic, such as form, color, and function.
Hit and lead compounds	Compounds with a certain set of desired properties (e.g., activity on target, cell toxicity, reactivity against nucleophiles, synthetic variability, efficacy in model animals). Lead compounds have more of the desired properties than hit compounds. The precise criteria are specifically defined by each drug discovery unit.
Drug	A substance which when absorbed in a living organism modifies one or more of its functions.
Off-label use	The practice of using a drug for a purpose outside the scope of its approved label.

Calculation of Constitutive Properties and Assignment to Phenotypic Classes

For the area of human pharmaceuticals, a large number of computational methods has been developed to differentiate between drug-like and nondrug-like [12, 13], as well as between leads and drugs [14]. The Ro5 was formulated to address issues

such as absorption [15], as an aid to exclude compounds that are likely to be less interesting to pursue. In the human pharmaceutical field, the oral delivery route is paramount. In the veterinary pharmaceutical field, various delivery routes are in use, according to indication, species, and costs. We were interested in the question of whether relationships could be obtained from the constitutive properties of registered veterinary pharmaceuticals, calculated by analogy to Lipinski, which could guide the selection of compounds for property determination. The chemical structures of active pharmaceutical ingredients (APIs) of veterinary drugs constitute the range of proven genotypes. A list of APIs registered for veterinary use was compiled from public sources [16] (Green Book, FDA Approved Animal Drug Products Database. FDA Center for Veterinary Medicine. http://www.fda.gov/cvm/greenbook.html. Last accessed Jan. 31, 2008; Tierarzneimittelkompendium der Schweiz. http://www.vetpharm.uzh.ch/perldocs/index_t.htm. Last accessed Jan. 31, 2008.).

Their number (355) is approximately only 20% of the number of APIs registered for human pharmaceutical use. APIs of drugs with an off-label use in the veterinary field were not considered. Starting from this set of chemical structures, we generated the values for the molecular weight (MW), the calculated distribution coefficient in 1-octanol/water, the count of oxygen and nitrogen atoms (ON), the number of acceptors (Acc), the number of donors (Don), the number of rotatable bonds (RotB), the ring count (Ring), and the three-dimensional dependent polar surface area (PSA) as additional descriptions of the genotype [17–19] (ClogP and PSA were calculated using SYBYL 7.3, Tripos International, St. Louis, Mo., USA).

The ON count was originally used by Lipinski to describe the number of acceptors. Not every oxygen or nitrogen constitutes an acceptor. This is taken into account by more recent calculation programs. These genotypes were assigned to the following phenotypic classes:

(i) Anthelmintics, antiprotozoals, insecticides, acaricides
Insecticides and acaricides partly overlap to form the group of ectoparasiticides. All classes together form the antiparasitics.

(ii) Antibacterials

(iii) Oral, parenteral (e.g., injectable, implant), topical or transdermal systemic route of application
Topical route of application means any drug delivery to the surface of the animal, with the drug exerting its pharmacological activity on the surface of the animal. It needs to be considered that a proportion of the drug may still be absorbed without leading to pharmacologically active dose levels. Transdermal means that the API is systemically available at a pharmacologically active dose level after topical application. Both topical and transdermal routes of application do not exclude oral uptake via licking or grooming behavior. While for topical administration, oral uptake reduces the availability of the API at the site of action accidentally, the ingestion of the API from transdermally applied drugs often contributes to the systemic availability of the API and is deliberately accepted.

Since the relevance of uptake of the API via grooming is not fully elucidated for most of the investigated drugs, and as such uptake varies greatly between species and individual animals, it was not further considered in the analysis. Here, we considered the route of application as a surrogate for absorption. This approach was chosen because veterinary drugs usually target a range of species, and due to physiological species differences, absorption data in various targeted species are not comparable. For the purpose of this investigation, we used the route of application as described in the specifications of the respective registered products. We should note that this does not mean that other routes would not work. A drug might be registered for topical application for reasons of convenience, or on account of the targeted pathogen(s), or due to toxicity considerations

(iv) Natural or non-natural

We use the expressions natural and non-natural instead of nonsynthetic or synthetic, which are sometimes used synonymously, as these properties relate to the origin of the chemical structure, not to the way of creation of the compound. APIs of non-natural origin were analyzed as a subset and compared to the whole data set.

Determination of Physical Properties and Assignment to Phenotypic Classes

To relate the above classifications to property space, we measured selected physicochemical properties of a subset of our API list. The compound set used for the physicochemical profiling comprised 292 out of the 355 API structures on our list, since not all of the API compounds could be obtained physically. Early physicochemical profiling in the process from hit compound to lead compound has become standard in the human pharmaceutical industry [20, 21]. We are not aware of similar approaches in the veterinary field. Properties that are broadly accepted to influence absorption *in vivo* are lipophilicity [22], aqueous solubility [23], and membrane permeability [24]. We chose measurements methods which would allow sufficient throughput: Lipophilicity, as expressed by logP, was determined as the water/octanol partitioning coefficient using an HPLC-based method [25]; solubility at pH 7.4 was determined nephelometrically [26]; and for membrane permeability the PAMPA double-sink protocol was used, with a pH value of 7.4 on both the donor and receiver side [27, 28]. We chose the PAMPA assay in preference to cell-based methods like CACO-2 [29], MDCK [30] or 2/4/A1 [31], because we consider passive permeability to be a critical and common property of antiparasitic drugs. In view of the variety of targeted pathogens, host species and application routes, a non-cell-based, artificial method like PAMPA can be expected to yield more consistent data than CACO-2, which resembles more the human *in vivo* situation.

Statistical Analysis of Genotypic and Phenotypic Data

We performed a statistical analysis aiming either to confirm existing assumptions on the relationship between structure/genotype and properties/phenotype, for example, from literature or in-house knowledge, or to find new relationships. The statistical analysis was limited by the relatively small number of registered veterinary drugs. We analyzed data for a total of 355 drugs, whereas the number of marketed human drugs is much greater, as shown by the analysis of 1729 human drugs [32]. Antibacterials form the largest part with 101 drugs, followed by anthelmintics (53), ectoparasiticides (48), and antiprotozoals (44) (Table 9.1; Figure 9.2).

Antibacterials have the highest mean molecular weight and the highest mean PSA value, whereas their mean clogP is the lowest. Ectoparasiticides, which include acaricides and insecticides, form the group with the lowest mean PSA, highest mean clogP, and lowest mean number of H-bond donors (Figure 9.3).

A comparison with other published data on insecticides [33, 34] shows that veterinary ectoparasiticides on average have higher PSA values and more H-bond donors and acceptors than compounds used as insecticides for agrochemical purposes [35] (Table 9.2). All groups show a relatively broad distribution of properties, with antibacterials being the broadest. Taking MW as an example, their 90th percentile for MW is 843, and for the ON count it is 18, whereas for the total set of compounds these percentiles are 621 and 13, respectively. A distribution plot of MW and ON for antibacterials shows the higher proportion of high-MW and high-ON compounds for antibacterials. (Figure 9.4).

It was observed previously that compounds of natural and non-natural origin differ in their properties [36, 37]. Our analysis revealed that antibacterials have the smallest percentage of non-natural compounds, whereas anthelmintics have the highest (Table 9.3). The analysis of the distribution according to structural origin showed that the broad property distribution is caused almost exclusively by APIs of natural origin. When calculating the molecular weight values for non-natural

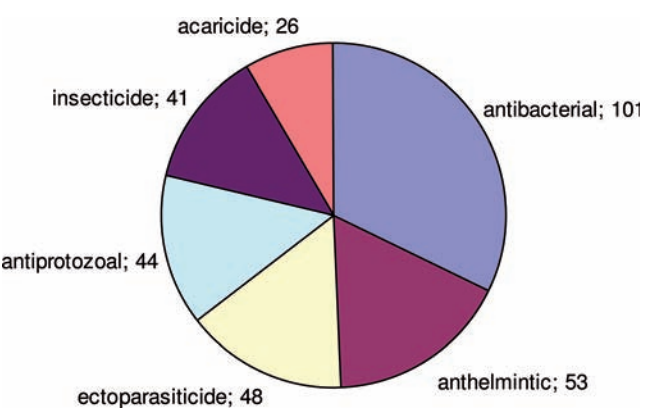

Figure 9.2 Distribution of APIs across indications.

Table 9.1 Number of APIs populating the indication and structural origin classes, and the mean, the 0–100th, 5–95th and 10–90th percentiles of the descriptor values.

Class	Origin	*n*	MW [g/mol]	clogP	ON	Acc	Don	RotB	Ring	PSA×10^{20} [m^2]
		mean								
All	All	355	387.6	2.1	6.6	5.1	2.7	6.2	2.8	156.8
	Non-natural	222	295.0	2.5	4.6	3.3	1.9	4.4	2.1	123.3
Anthelmintic	All	53	381.3	3.5	5.8	4.6	2.2	4.3	2.8	131.4
	Non-natural	45	314.5	3.4	4.6	3.2	1.8	3.4	2.2	122.1
Insecticide	All	41	406.2	4.2	5.9	5.3	1.2	6.4	2.5	111.4
	Non-natural	29	308.8	3.2	4.9	4.1	1.1	5.4	1.4	110.5
Acaricide	All	26	462.3	4.8	6.3	5.7	1.4	6.6	3.2	103.9
	Non-natural	14	301.4	3.5	4.3	3.3	1.0	5.2	1.4	88.3
Ectoparasiticide	All	48	397.8	4.3	5.7	5.0	1.3	6.1	2.4	106.4
	Non-natural	34	309.7	3.4	4.7	3.8	1.1	5.4	1.4	102.8
Antiprotozoal	All	44	399.4	1.9	7.7	5.8	2.8	7.1	2.8	190.9
	Non-natural	33	298.2	2.0	5.8	3.8	2.3	4.7	2.2	175.2
Antibacterial	All	101	493.4	0.1	10.7	8.3	4.6	8.7	3.2	242.0
	Non-natural	39	286.1	0.9	6.0	5.0	2.3	3.8	2.4	172.5
		0–100%								
All	All	355	82–1674	−24–13	0–44	0–32	0–20	0–43	0–11	0–824
	Non-natural	222	82–663	−9–9	0–12	0–8	0–10	0–17	0–5	0–331
Anthelmintic	All	53	86–914	−7–9	1–16	0–14	0–11	0–16	0–8	27–409
	Non-natural	45	86–663	−1–9	1–11	0–7	0–4	0–14	0–4	27–307
Insecticide	All	41	166–914	−2–8	3–15	2–14	0–4	0–11	0–8	20–277
	Non-natural	29	166–511	−2–6	3–7	2–6	0–3	0–11	0–3	20–277
Acaricide	All	26	201–914	1–8	2–15	0–14	0–4	1–11	0–8	9–198

(*Continued*)

Table 9.1 (*Continued*)

Class	Origin	*n*	MW [g/mol]	clogP	ON	Acc	Don	RotB	Ring	PSA × 10^{20} [m^2]
	Non-natural	14	201–506	1–6	2–6	0–6	0–3	1–11	0–3	9–198
Ectoparasiticide	All	48	166–914	−2–8	2–15	0–14	0–4	0–11	0–8	9–277
	Non-natural	34	166–511	−2–6	2–7	0–6	0–3	0–11	0–3	9–277
Antiprotozoal	All	44	141–1297	−9–9	2–29	1–23	0–14	1–20	1–8	68–798
	Non-natural	33	141–461	−9–9	2–12	1–8	1–6	1–16	1–5	68–331
Antibacterial	All	101	140–1674	−24–6	2–44	0–32	1–20	0–43	1–11	23–824
	Non-natural	39	140–399	−2–5	2–9	0–8	1–5	0–7	1–5	23–317
		5–95%								
All	All	355	187–851	−3–6	2–17	1–13	0–8	1–16	1–5	26–347
	Non-natural	222	171–420	0–6	2–8	0–6	0–4	1–10	1–4	23–277
Anthelmintic	All	53	200–874	0–7	2–14	0–14	0–4	0–11	1–7	37–266
	Non-natural	45	200–441	1–7	2–8	0–6	0–3	0–8	1–4	37–234
Insecticide	All	41	201–875	1–7	3–14	2–14	0–3	1–11	0–7	30–198
	Non-natural	29	194–456	1–6	3–6	2–6	0–3	0–11	0–2	32–239
Acaricide	All	26	212–893	2–8	2–14	0–14	0–3	2–11	0–7	26–186
	Non-natural	14	206–461	2–6	2–6	0–5	0–2	1–9	0–3	19–181
Ectoparasiticide	All	48	204–874	1–7	3–14	1–14	0–3	1–11	0–7	27–196
	Non-natural	34	197–480	1–6	3–6	0–6	0–3	1–10	0–2	23–220
Antiprotozoal	All	44	193–857	−4–7	3–17	1–15	1–6	1–17	1–7	81–329
	Non-natural	33	184–421	−2–7	3–8	1–6	1–5	1–11	1–4	77–298
Antibacterial	All	101	225–1203	−5–5	5–29	3–19	1–14	2–26	2–5	107–535
	Non-natural	39	168–386	−1–4	4–8	2–7	1–4	1–6	1–4	80–241
		10–90%								
All	All	355	214–621	−1–5	2–13	1–11	0–5	2–14	1–5	37–287
	Non-natural	222	198–390	0–5	2–7	1–6	0–3	1–8	1–3	29–225

Anthelmintic	All	53	208–658	2–6	2–12	1–11	0–3	1–10	1–5	46–192
	Non-natural	45	205–410	2–5	2–7	1–5	0–3	0–6	1–3	41–189
Insecticide	All	41	221–770	1–7	3–12	3–11	0–3	3–10	1–7	51–172
	Non-natural	29	207–398	1–6	4–6	3–6	0–3	2–9	0–2	49–180
Acaricide	All	26	220–874	2–7	3–14	1–14	0–3	3–11	1–7	31–166
	Non-natural	14	212–405	2–6	2–6	0–5	0–2	2–8	0–3	27–156
Ectoparasiticide	All	48	220–679	1–7	3–10	2–10	0–3	3–10	1–6	43–164
	Non-natural	34	212–420	1–6	3–6	2–6	0–3	1–9	0–2	37–162
Antiprotozoal	All	44	215–727	−1–6	4–12	2–11	1–5	2–16	1–5	92–284
	Non-natural	33	203–413	0–5	3–8	2–6	1–4	2–8	1–3	88–280
Antibacterial	All	101	255–843	−4–4	5–18	4–14	1–11	3–20	2–5	131–417
	Non-natural	39	220–360	0–3	5–8	2–6	2–3	2–6	1–4	126–204

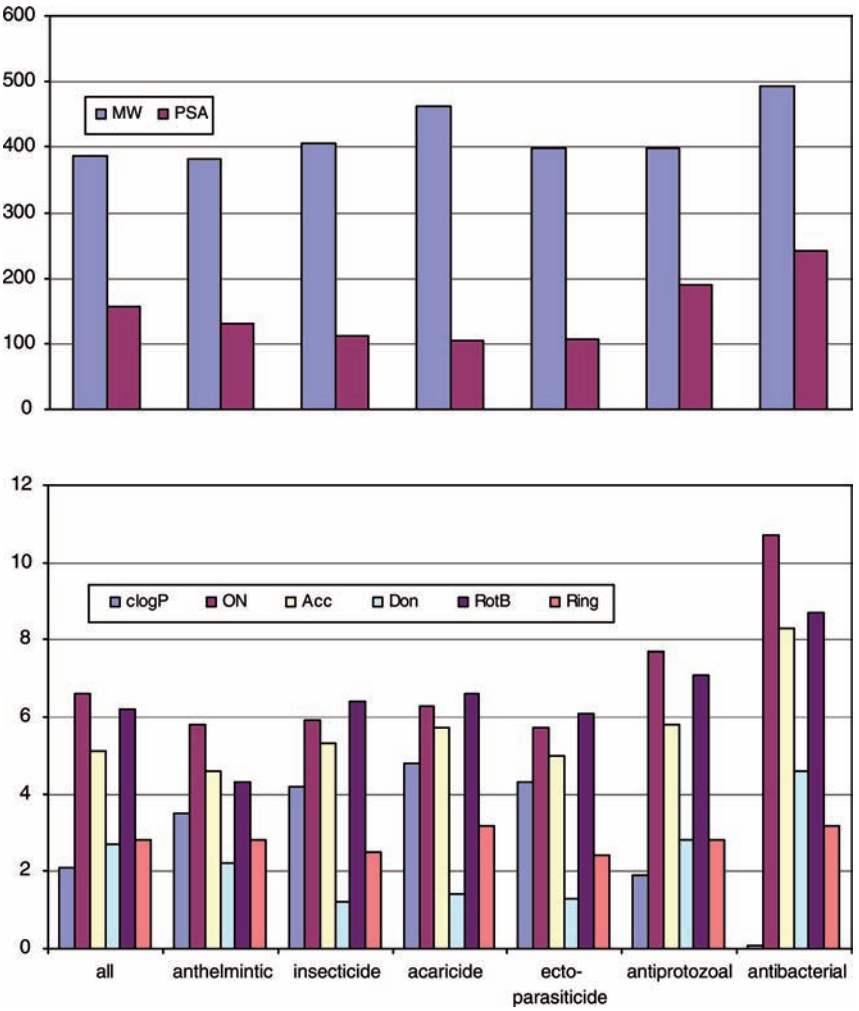

Figure 9.3 Calculated mean values for constitutive properties. Y-axis units were omitted for the purpose of clarity, and can be found in the supplementary tables.

compounds only, the 100th percentile value for ectoparasiticides goes down from 914 to 511, for antiprotozoals from 1297 to 461, and for antibacterials from 1674 to 399 (Table 9.1). Likewise, the mean MW and the number of rotatable bonds go down across all indications. The 5th to 95th percentiles for non-natural compounds, with the exception of clogP, lie in a drug-like range, according to Lipinski's Ro5 (Figure 9.5).

In general, differences between indications tend to be much less pronounced if non-natural compounds are considered only, for example, the MW is essentially

Table 9.2 Comparison of the distribution range of descriptors of ectoparasiticides with agrochemical insecticides.

Dataset	Percentile	*n*	MW [g/mol]	logP	ON	Don	PSA × 10^{20} [m^2]
Insecticides, Clarke [34]	10th 90th		210–500	0.9–6.6 (ElogP)			
Insecticides, Tice [35]	0th 100th	237	136–905	−1.8–8.4 (alogP)	0–16	0–5	0–139
	5th 95th	237	185–502	0–6.4	1–7	0–2	14.8–83
	mean	237	322	3.5	4.2	0.4	49.8
Veterinary ectoparasiticides	0th 100th	48	166–914	−2–8 (clogP)	2–15	0–4	9–277
	5th 95th	48	204–874	1–7	3–14	0–3	27–196
	mean	48	397	4.2	5.6	1.2	106.4

the same, as reflected by the means and the 10th to 90th percentile values. A difference in PSA and clogP can be observed between non-natural anthelmintics and ectoparasiticides on the one hand, and antiprotozoals and antibacterials on the other hand, the former having significantly lower mean PSA values (122/103 compared to 175/173) and higher clogP values (3.4/3.4 compared to 2.0/0.9; Figure 9.6).

It is worthwhile to consider two extreme API structures of natural structural origin: the avermectins, belonging to the class of macrocyclic lactones, with ectoparasitic and anthelmintic activity, and the ionophores like the salinomycins, belonging to the class of polyethers, and used as antiprotozoals. Both form extensive patterns of intramolecular H-bonding which can not be reflected appropriately by descriptors like the ones used in this analysis.

A similar picture evolves from the comparison of the different application forms (Table 9.4). The range covered by non-natural compounds is significantly smaller than the whole data set. Natural compounds tend to have higher molecular weights, PSAs, and numbers of donors and acceptors, irrespective of their application route, but no clear trend is seen with regard to clogP. All routes show remarkably similar constitutive properties, with the exception of transdermal compounds. Thus, their mean PSA is smaller by $40 \times 10^{-20}\,m^2$ to $60 \times 10^{-20}\,m^2$ compared to the other applications ($40 \times 10^{-20}\,m^2$ for non-natural compounds), the number of donors and rotatable bonds is smaller, and the ring count is higher by one (Figure 9.7).

The greater restrictions apparently imposed upon the transdermal route are striking. However, we have to point out that this group is by far the smallest, so statistical comparisons require caution. The comparison of calculated results for human APIs and our data for veterinary APIs shows that the values for injectables are clearly distinct from drugs applied orally and topically in the human set, whereas this distinction cannot be seen between veterinary parenterals and oral or topical

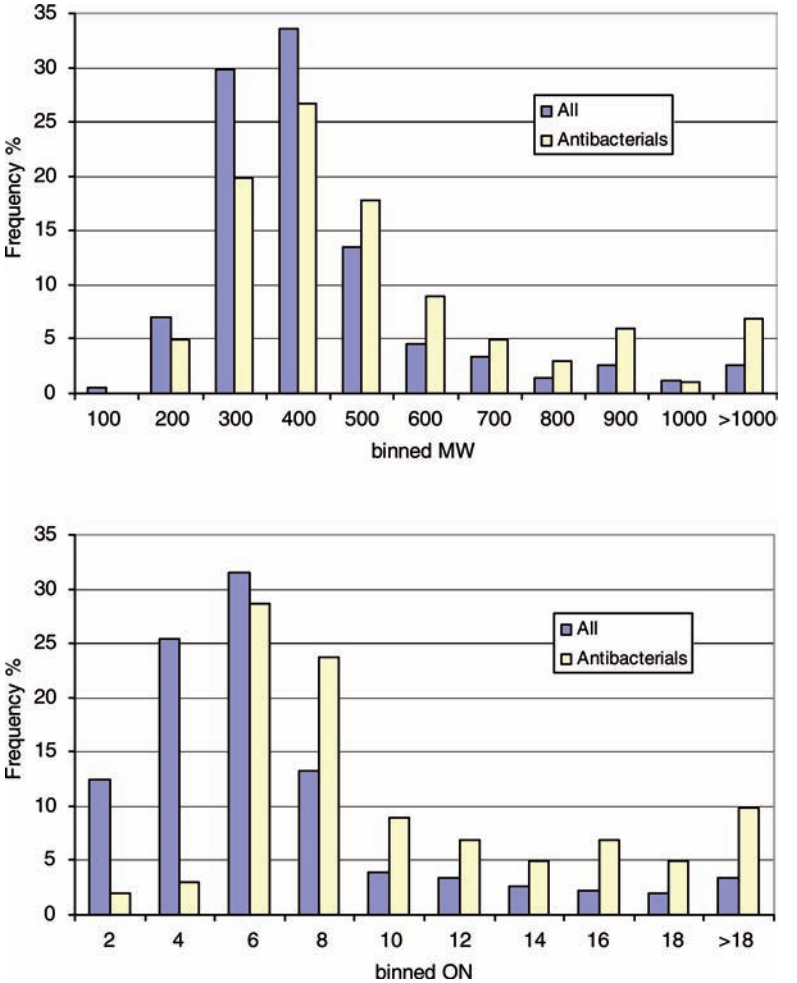

Figure 9.4 Distribution plot of MW and ON for antibacterials versus all compounds.

Table 9.3 Percentage of natural and non-natural structural origin.

Class	*n* total	% Natural	% Non-natural
All	355	37.5	62.5
Anthelmintic	53	15.1	84.9
Insecticide	41	31.7	68.3
Acaricide	26	46.2	53.8
Ectoparasiticide	48	29.2	70.8
Antiprotozoal	44	27.3	72.7
Antibacterial	101	61.4	38.6
Parenteral	185	46.5	53.5
Oral	197	32.0	68.0
Topical	74	41.9	58.1
Transdermal	17	41.2	58.8

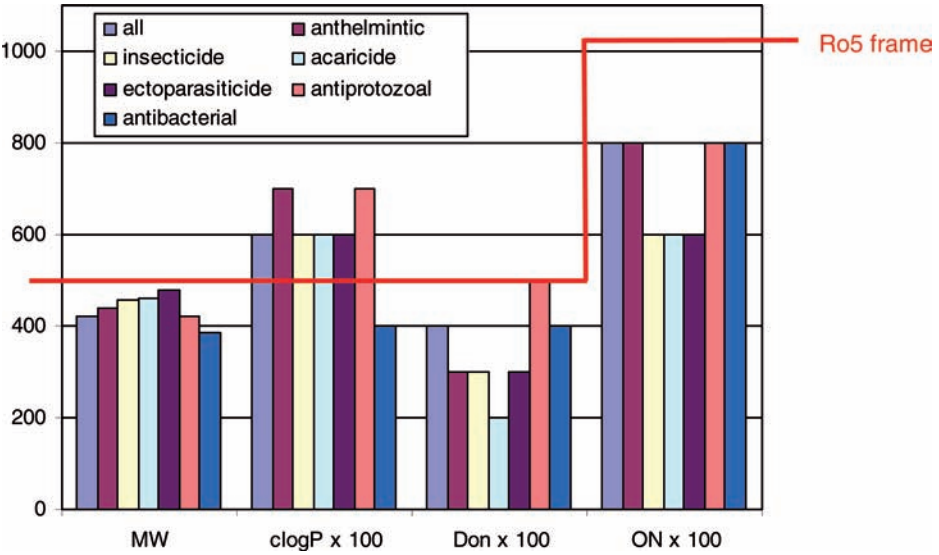

Figure 9.5 Constitutive property values for the 95th percentile for non-natural compounds, as compared to Lipinski's Ro5 values. Y-axis units were omitted for the purpose of clarity, and can be found in the supplementary tables. clogP, Don and ON values were multiplied by 100 to fit the graphic.

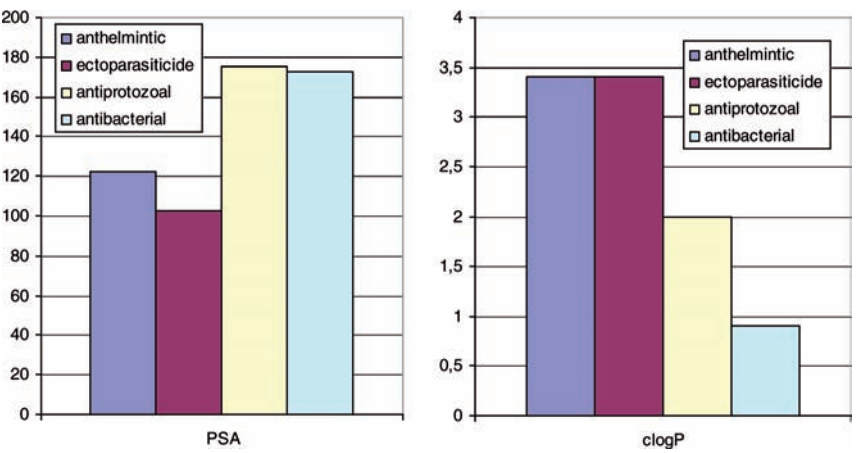

Figure 9.6 Differences in PSA and clogP between non-natural ectoantiparasitics/anthelmintics and antiprotozoals/antibacterials. Y-axis units were omitted for the purpose of clarity, and can be found in the supplementary tables.

Table 9.4 Comparison of different application forms.

Class/application	Origin	*n*	MW [g/mol]	clogP	ON	Acc	Don	RotB	Ring	PSA × 10^{20} [m^2]
		Mean								
Parenteral	All	185	380.5	1.5	6.4	4.8	3.0	6.0	2.9	161.2
	Non-natural	98	293.0	2.1	4.2	2.9	2.1	4.2	2.2	114.4
Oral	All	197	379.5	2.1	6.8	5.3	2.8	5.8	2.7	160.6
	Non-natural	134	302.0	2.4	5.1	3.8	1.9	4.1	2.2	137.7
Topical	All	74	433.1	2.8	7.4	5.6	3.1	7.9	2.5	164.4
	Non-natural	43	297.3	3.4	4.4	3.3	1.4	5.6	1.6	103.9
Transdermal	All	17	463.4	3.5	6.5	5.6	1.9	4.5	3.9	102.2
	Non-natural	11	298.4	2.7	3.6	2.2	1.1	2.4	2.4	66.8
		0–100%								
Parenteral	All	185	82–1674	−24–9	0–44	0–28	0–18	0–43	0–8	0–824
	Non-natural	98	82–626	−9–9	0–8	0–8	0–6	0–13	0–5	0–331
Oral	All	197	86–1423	−7–9	1–33	0–20	0–17	0–30	0–7	0–609
	Non-natural	134	86–663	−4–9	1–12	0–8	0–4	0–16	0–5	0–327
Topical	All	74	166–1665	−6–13	0–37	0–25	0–20	0–30	0–10	0–709
	Non-natural	43	166–505	−2–6	1–10	0–6	0–10	0–17	0–4	9–317
Transdermal	All	17	204–914	−3–7	1–15	0–14	0–4	0–11	1–8	6–191
	Non-natural	11	204–506	−3–6	1–6	0–5	0–3	0–6	1–4	6–161
		5–95%								
Parenteral	All	185	185–805	−4–5	2–17	1–13	0–8	1–16	1–5	27–398
	Non-natural	98	170–396	−1–5	2–7	1–6	0–4	1–8	1–4	21–277
Oral	All	197	199–823	−2–6	2–16	1–13	0–8	1–16	1–5	32–320
	Non-natural	134	190–419	0–6	2–8	1–6	0–3	0–8	1–4	24–276

Topical	All	74	195–1023	−3–7	2–22	0–17	0–12	1–21	0–5	23–556
	Non-natural	43	179–435	0–6	2–7	0–6	0–3	1–11	0–3	22–275
Transdermal	All	17	216–902	1–6	2–14	0–14	0–3	1–10	1–7	24–173
	Non-natural	11	211–430	−1–5	1–6	0–5	0–3	0–5	1–4	16–148
		10–90%								
Parenteral	All	185	223–583	−2–4	2–12	1–10	1–6	2–13	1–5	37–287
	Non-natural	98	194–381	0–5	2–7	1–5	1–3	2–7	1–4	28–212
Oral	All	197	221–632	−1–5	3–13	2–11	1–6	2–12	1–4	57–285
	Non-natural	134	205–394	0–5	2–7	1–6	1–3	1–7	1–3	38–221
Topical	All	74	222–681	−2–6	2–15	1–13	0–9	2–16	1–4	31–334
	Non-natural	43	199–362	1–5	2–6	1–6	0–3	1–11	0–3	25–226
Transdermal	All	17	221–885	2–6	2–14	1–14	0–3	1–10	1–7	31–164
	Non-natural	11	217–353	1–5	2–6	0–5	0–3	1–3	1–3	26–134

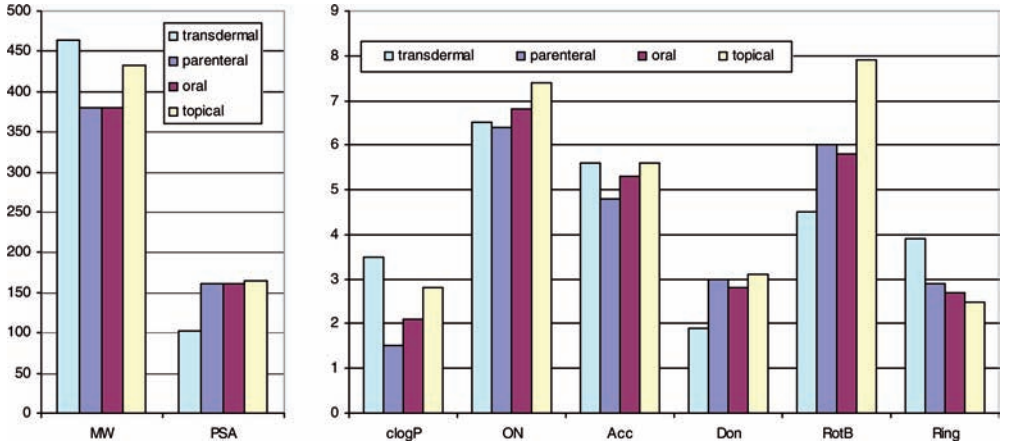

Figure 9.7 Transdermal compounds (pale blue) display different constitutive properties from the other categories. Y-axis units were omitted for the purpose of clarity and can be found in the supplementary tables.

compounds (Table 9.5). As noted previously, the structural origin of the veterinary compounds has the strongest influence on the values.

Comparison of Constitutive Properties and Measured Values

On comparison of the findings on the constitutive properties described above with the measured values for logP and permeability, a different picture emerges (Table 9.6). In spite of the low number of compounds that were physically available for testing, clear distinctions could be pinpointed:

(i) The different indications are clearly distinct in their lipophilicity, with antibacterials being the least lipophilic (mean $\log P = 0.7 \pm 1.8$), whereas ectoparasiticides show the highest mean lipophilicity (mean $\log P = 3.5 \pm 1.7$). Second most lipophilic are anthelmintics (mean $\log P = 2.7 \pm 1.8$).

(ii) The application forms also relate to a difference in lipophilicity: Topical drugs are most lipophilic with 2.7 ± 1.8 for logP, whereas parenteral application is associated with the lowest logP.

(iii) The logP values for compounds of non-natural origin differ from the values of the complete data set by less than half a log unit, and the differences are thus negligible. The only exception is the 90th percentile value for transdermal application, where the number of available compounds was the smallest of all groups. This finding is in clear contrast to that for the calculated clogP, and

Table 9.5 Different application forms in human and veterinary drugs.

Data set	Route	Percentile	*n*	MW [g/mol]	clogP	ON	Acc	Don	RotB	Rings	PSA × 10^{20} [m^2]
Marketed human drugs, Vieth [32]	Oral	0th 100th	1193	74–1449	−7.6–20.2	0–33	0–17	0–18	0–40	0–10	0–447
		5th 95th	1193	164–589	−1.9–6.3	2–12	0–7	0–4	1–12	1–5	13–169
		Mean	1193	343	2.3	5.5	3.2	1.8	5.4	2.6	78
	Injectable	0th 100th	308	46–5826	−19.9–10	0–144	0–75	0–75	0–156	0–11	0–879
		5th 95th	308	163–1297	−5.0–5.8	2–30	0–15	0–16	1–42	0–7	20–416
		Mean	308	558.2	0.6	11.3	6.2	4.7	12.7	3.2	143.6
	Topical	0th 100th	112	60–1423	−11.3–10	0–33	0–23	0–21	0–35	0–6	0–557
		5th 95th	112	130–505	−2.4–6.7	1–10	0–7	0–4	0–13	0–5	4–156
		Mean	112	368.5	2.9	5	3.2	1.8	5.3	2.9	75.4
This analysis	Oral	0th 100th	197	86–1423	−7–9	1–33	0–20	0–17	0–30	0–7	0–609
		5th 95th	197	199–823	−2–6	2–16	1–13	0–8	1–16	1–5	32–320
		Mean	197	379.5	2.1	6.8	5.3	2.8	5.8	2.7	160.6
	Parenteral	0th 100th	185	82–1674	−24–9	0–44	0–28	0–18	0–43	0–8	0–824
		5th 95th	185	185–805	−4–5	2–17	1–13	0–8	1–16	1–5	27–398
		Mean	185	380.5	1.5	6.4	4.8	3.0	6.0	2.9	161.2
	Topical and Transdermal	0th 100th	87	166–1665	−6–13	0–37	0–25	0–20	0–30	0–10	0–709
		5th 95th	87	199–923	−3–7	2–19	0–16	0–11	1–20	0–6	22–499
		Mean	87	437.8	2.9	7.2	5.6	2.9	7.4	2.7	153.8

Table 9.6 Measured logP and permeability values (PAMPA). Mean values are supplemented by standard deviations and number of measured compounds per category.

	log P		Permeability $\times 10^8$ [m/s]	
Phenotypic class	**All**	**Non-natural**	**All**	**Non-natural**
Mean				
All	1.8 ± 1.8 (259)	1.8 ± 1.8 (173)	18.9 ± 21.1 (205)	18.9 ± 21.4 (136)
Anthelmintic	2.7 ± 1.8 (38)	2.5 ± 1.8 (34)	17.0 ± 17.1 (33)	18.3 ± 17.7 (29)
Ectoparasiticide	3.5 ± 1.7 (39)	3.2 ± 1.8 (29)	17.4 ± 16.5 (27)	22.2 ± 17.1 (17)
Antiprotozoal	1.9 ± 1.9 (29)	1.7 ± 1.9 (22)	10.3 ± 12.7 (26)	10.0 ± 12.6 (23)
Antibacterial	0.7 ± 1.8 (76)	0.6 ± 1.7 (34)	8.0 ± 11.0 (57)	8.1 ± 10.1 (32)
Parenteral	1.3 ± 1.6 (135)	1.4 ± 1.5 (75)	21.0 ± 24.1 (101)	21.0 ± 26.3 (54)
Oral	1.6 ± 1.9 (207)	1.6 ± 1.8 (107)	16.0 ± 18.1 (120)	15.2 ± 17.7 (93)
Topical	2.7 ± 1.8 (61)	3.0 ± 1.6 (38)	18.7 ± 19.9 (47)	25.2 ± 21.1 (28)
Transdermal	2.3 ± 1.8 (13)	1.9 ± 1.9 (9)	18.9 ± 18.1 (8)	21.0 ± 21.1 (4)
0–100%				
All	−2.0–5.5	−2.0–5.2	0.0–101.2	0.0–101.2
Anthelmintic	−1.0–4.9	−1.0–4.9	0.0–58.7	0.0–58.7
Ectoparasiticide	−1.3–5.5	−1.3–5.0	0.0–52.8	1.6–52.8
Antiprotozoal	−2.0–5.2	−2.0–5.2	0.0–38.4	0.0–38.4
Antibacterial	−2.0–5.2	−2.0–5.2	0.0–49.3	0.0–36.5
Parenteral	−2.0–4.6	−2.0–4.6	0.0–101.2	0.0–101.2
Oral	−2.0–4.9	−2.0–4.9	0.0–73.4	0.0–73.4
Topical	−2.0–5.5	−0.9–5.0	0.0–85.6	0.0–85.6
Transdermal	−2.0–4.8	−2.0–4.8	0.0–52.8	5.8–52.8
10–90%				
All	−0.8–4.3	−0.7–4.3	0.0–52.1	0.0–51.7
Anthelmintic	−0.4–4.6	−0.4–4.5	0.6–40.1	1.0–42.6
Ectoparasiticide	0.9–5.0	0.5–4.7	1.8–41.2	4.1–43.2
Antiprotozoal	−0.7–4.2	−0.9–4.2	0.0–33.5	0.0–33.2
Antibacterial	−2.0–2.7	−1.0–2.5	0.0–20.6	0.0–21.1
Parenteral	−1.0–3.2	−0.6–3.2	0.0–58.3	0.0–61.2
Oral	−1.0–4.2	−0.8–4.2	0.0–48.4	0.0–40.1
Topical	0.4–4.9	0.8–4.5	0.6–43.3	2.4–49.7
Transdermal	0.7–4.5	0.1–3.6	4.1–42.9	5.9–42.8

likewise, the measured permeabilities are almost identical between the complete set and the non-natural subset. The permeabilities in themselves are quite high; the means for all therapeutic areas $\geq 10^{-6}$ cm/s, which qualifies the compounds as being highly permeable [38]. Antibacterials and compounds for oral application have the smallest permeability values, which is not surprising, since most β-lactams and similar compounds are absorbed by active transport.

The Potency–Solubility–Permeability Triad and Veterinary Pharmaceuticals

In human pharma, permeability and aqueous solubility are often used in combination to predict oral bioavailability [39]. These are also known as the triad of potency, solubility, and permeability [40]. The main idea is that high permeability can compensate for low solubility, and vice versa. A statistical analysis of aqueous solubility in our case is limited by the nephelometric method we used, which yields information on kinetic solubility. Instead of statistical calculation, a binning in categories followed by graphical analysis was chosen. Figure 9.8a, b plots solubility and permeability against each other and codes them for indication and application. In view of the limited number of compounds, the antiparasitic classes are grouped together. With a few exceptions, all compounds fit into the picture of the triad mentioned above. They show either high solubility or high permeability or both, whether they are applied orally or not. Two compounds that seem to contradict this are really quite exceptional (Figure 9.8a): One is ivermectin (blue arrow), which is extremely potent and usually dosed below 1 mg/kg; the other is iodochloroquine (red arrow), which is used as an intestinal or topical disinfectant and is not absorbed.

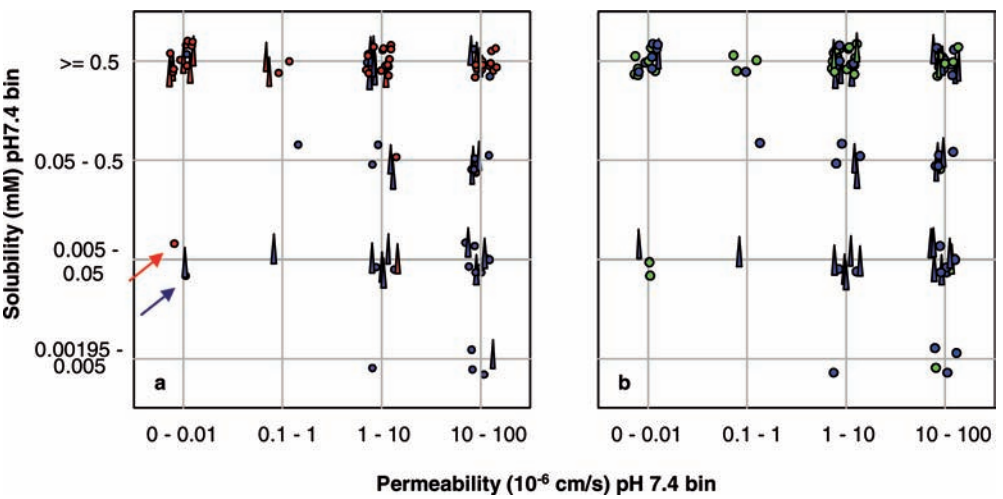

Figure 9.8 Plot of aqueous solubility against PAMPA permeability. A, red: antibacterials; blue: antiparasitics; circles: approved for oral application; triangles: approved for other routes than oral. B, green circles: antiparasitics approved for parenteral application; blue triangles: antiparasitics approved for topical application. Red arrow pointing at iodochloroquine, blue arrow at ivermectin.

It is evident that antibacterials and antiparasitics behave differently in this respect. Whereas antibacterials almost exclusively fall into the group with highest solubilities, their permeabilities vary broadly. This again reflects the fact that many antibacterials are absorbed via active transport. Antiparasitics, on the other hand, fall generally into the bins with high permeability, whereas their solubilities cover all the bins. This implies that aqueous solubility is a limiting factor for antibacterials but not for antiparasitics, whereas high permeability is a characteristic for antiparasitics.

An analogous coding for parenteral and topical application of antiparasitics (Figure 9.8b) yields a similar picture. Compounds that are applied parenterally have high solubility, whereas topically applied drugs have high permeability.

Conclusions

In this study, we aimed to identify characteristics for the genotypic descriptors, calculated by analogy to Lipinski, which could be translated into the phenotypic property space, as exemplified by permeability and lipophilicity values, and vice versa. We learned that the genotype descriptors mainly relate to the phenotypic class of the structural origin of the APIs, either natural or non-natural. In contrast, measured permeability and lipophilicity values as descriptors of the property space allow for differentiation between the phenotypic classes (like different application forms), but they are independent of the structural origin of the API.

Translation between these simple structural descriptors and property space does not seem feasible. We assume that these structural descriptors do not adequately describe and abstract the API for the purpose of translation between the two spaces, structure and property. Many of the compounds of natural structural origin are rather big macro- or polycyclic compounds with very distinct conformations, which apparently can not be adequately described by the simple descriptors used so far. To overcome this, one could look instead at other descriptors than those used, which take conformation into account, and which would lead to a loss of the almost intuitive understanding of the simple descriptors. This extension into conformational space was discussed just recently [41]. We encourage the continued search for descriptors which allow a correlation of the structural space with the property space – between the genotype and phenotype of pharmaceuticals. Veterinary drug discovery, and pharmaceutical research in general, will greatly profit from the resolution of this apparent dichotomy.

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10
Recruiting the Host Defense Mechanisms: Roles for Vaccines and Chemotherapeutics

*Theo P.M. Schetters**

Abstract

For almost a century, an industrial approach has been adopted to purify effective compounds from natural sources and to discover new drug targets, with the ultimate goal of synthesizing effective drugs. Drugs can be used to directly kill the organism, to limit the proliferation such that the infected subject is aided in the control of the infection, or administered together with a live vaccine to actively vaccinate the host. For instance, vaccination against the protozoan blood parasite *Theileria parva* is done with a cover of long-acting tetracycline given simultaneously with the vaccine parasite strain. Preferably, vaccines are used without drugs. A multitude of approaches has been successfully developed from administration of a low dose of virulent organisms to vaccination with attenuated live vaccines, non-live subunit vaccines and recombinantly produced vaccines. Most of the non-live vaccines need an adjuvant to be effective; and also here a multitude of approaches have been used, from classic oil-based adjuvants to a sophisticated implementation of immunomodulating cytokines in vaccine formulations. This chapter provides an overview of vaccines and vaccination strategies developed for use in veterinary practice, with special attention to vaccines against veterinary parasites.

Ancient Cultural Practices

Control of infectious disease has been practiced since ancient times but has gone unrecognized for a long period of time as part of local tradition. With the development of the natural sciences and the subsequent increased understanding of complex biochemical pathways, many principles of traditional medicine have been discovered and exploited commercially. For instance, the development of the antimalarial drug quinine: this was isolated in 1817 from the bark of the cinchona

tree, but had been in use already in 1600 in an unextracted form in several parts of the world [1]. It is less evident that vaccination also has a long history. Usually reference is made to the discovery in 1796 of the British medic Jenner who used the cowpox virus to vaccinate against smallpox in humans. However, vaccination had its roots in variolation, a method in which the virulent *Variola* virus was inoculated into children to protect against smallpox. This practice became more widely known in Europe early in the eighteenth century and was more generally used in the Ottoman Empire. The oldest records of such practice are from traditional Chinese medicine [2, 3]. It should be realized that, at the time, the cause of many infectious diseases was unknown; the term virus (Latin for "poison, sap of plants, slimy liquid;" http://www.etymonline.com) was used to describe the material derived from smallpox. Also in parasitic diseases, inoculation with material from ulcers was used for inoculation. The best known example is leishmanization, inoculation of *Leishmania tropica* to protect against zoonotic cutaneous leishmaniosis [4]. The general introduction of such vaccination practices was hampered due to the high virulence of the inoculum.

Rational Vaccine Development

Development of Attenuation

Impetus to the rational development of safe and efficacious vaccines was given by Louis Pasteur and his collaborators. It was the era of the microscopic description of a large number of microbiological pathogens, many of which carry the name of their discoverer (Louis Pasteur → *Pasteurella*, Daniel Elmer Salmon → *Salmonella*, Albert Neisser → *Neisseria*, Victor Babeş → *Babesia*, Arnold Theiler → *Theileria*, etc.). Pasteur had observed that bacteria from a culture of *Pasteurella aviseptica* appeared less virulent upon prolonged cultivation, and that animals inoculated with this culture were protected against challenge with the virulent strain [2]. Since then, the general principle became to adapt the pathogen to *in vitro* culture and subsequently test the efficacy and safety by vaccination of experimental animals and subsequent challenge infection. Among these early successes were vaccines against tuberculosis in man (an attenuated strain of *Mycobacterium tuberculosis* called Bacille de Calmette et Guérin; BCG) and anthrax in sheep [3]. A major issue was the stability of the inocula the efficacy of which decreased upon storage. In 1897 it was found that when live *M. butyricum* was injected into experimental animals, lesions developed that sometimes resembled tubercles, but when the bacteria were incorporated into butter they evoked a cellular reaction almost identical with the tubercles caused by pathogenic tubercle bacilli; and in 1899 Grassberger reported that paraffin oil was far more effective [5].

The development of virus vaccines was lagging behind, mainly because the pathogens were not detectable by light microscopy in contrast to parasites, yeasts, and bacteria, although there was a sense that pathogens smaller than bacteria had to

exist. Viruses (bacteriophages) were first described by Felix d'Herelle in 1917 [6]. The rational development of viral vaccines required sterile cultivation techniques, and in 1931 the first cultures of influenza viruses in eggs were reported [7]. It took almost another two decades before propagation of polio virus on nucleated cells was successful [8]. From that time on, obtaining vaccine strains by attenuation of viruses by serial passage became a realistic option.

Development of Killed Vaccines and Adjuvants

Almost immediately after the discovery that attenuated bacteria could be used as a vaccine it was discovered that, in some cases, heat-killed bacteria were also effective [9]. Although this was effective in the case of typhoid fever and cholera, in other cases it was found that, after heat-inactivation, the protective activity was lost. Increasing the dose was not always possible because of severe adverse reactions that were due to bacterial toxins. At the time it was also known from pharmacology that relatively high doses of toxins (for instance strychnine) could be injected, provided these were suspended in oil, which was due to the slow release of the toxin from the oil [10]. This was employed to prepare bacterial vaccines that contained relatively high numbers of bacteria. These studies are considered among the first using oil-based adjuvants with the development of Freunds complete adjuvant (FCA) in 1937 as highlight [11]. It should be realized that it was not known what the mechanisms were that increased the efficacy of vaccination.

Immunology

The light microscope was crucial in the early days of microbiology. Koch and Neisser observed that white blood cells sometimes contained bacteria, which was interpreted that the bacteria had invaded these host cells. Eli Metchnikoff showed that, in fact, the leucocytes had engulfed the bacteria, a process that he called phagocytosis [2]. From the studies of Metchnikoff, it became clear that the white blood cells in some way specifically recognized the bacteria; they did not phagocytose each other. In addition the study of immunized animals revealed that specificity was sometimes associated with a heat-stable factor in serum (immunoglobulin; Behring and Kitasato 1890, cited in Ref. [2]). In retrospect these appeared to be the first experiments of a discipline later called immunology. Central in immunology became the principle of cognate interaction of foreign substances/entities by specific receptor–ligand interactions. These receptors are generally divided in two classes: the innate receptors and the immune receptors. Present-day vaccinologists try to find ways to exploit these principles to develop effective vaccines and vaccination protocols (sometimes involving the use of drugs; see below). In order to gain insight into these approaches it is necessary to understand the basic principles of the immune response.

Natural Immunity

Immunology defines a number of systems and responses, such as the complement system, the kinin system, the coagulation system, the acute phase response, humoral immune response, the cellular immune response, etc. Each of these systems comprises cells as well as soluble compounds released from cells. Over the years it became clear that these are merely models and that all systems play a role and interact with each other in the generation of a certain response [12]. Importantly, in each of these systems and at each point of time cognate interactions occur that involve pathogen associated molecular structures and host encoded ligands. Host molecules that interact with pathogen-derived molecular structures may be present in body fluids as soluble factors or cell-associated; the latter usually in the form of cell surface receptors (Table 10.1). Different stages of infection can be recognized: incubation period, adaptive immune response, outcome (recovery or chronic infection/death). The first line of defense mechanisms that are constitutively present belong to the innate immune system. These systems have a relatively broad spectrum of activity (e.g., proteolytic enzymes, polymorphnuclear granulocytes). More specific, tailor-made effector responses, take more time to develop. These responses belong to the

Table 10.1 Vaccination strategies in the control of parasitic infections.

Strategy	Parasite	Host	Reference
Low-dose infection	*Eimeria* species	Poultry	[32]
Infection and subsequent treatment	*Cowdria ruminantium*	Sheep	[27]
Infection and simultaneous treatment	*Theileria parva*	Cattle	[26]
Live attenuated	*Dictyocaulus viviparus*	Cattle	[37]
	Babesia bovis	Cattle	[31]
	B. bigemina	Cattle	[31]
	T. annulata	Cattle	[59]
	T. hirci	Sheep/goats	[60]
	Anaplasma marginale	Cattle	[34]
	Toxoplasma gondii	Sheep	[61]
	Eimeria species	Poultry	[32]
	C. ruminantium	Cattle	[27]
Killed	*Trichomonas foetus*	Cattle	[62]
	Neospora caninum	Cattle	[41]
	Giardia lamblia	Dog	[42]
	B. divergens	Cattle	[43]
	Leishmania brasiliensis	Man	[38]
Subunit	*B. canis*	Dog	[45]
	B. rossi	Dog	[46]
	Taenia species	Sheep	[47]
	B. divergens	Cattle	[48]
	L. infantum	Dog	[55]

adaptive immune system comprising antibody- and cytokine-producing lymphocytes and cytotoxic lymphocytes [12].

The Incubation Period

The incubation period is defined as the period of time before overt signs of disease, such as fever, become apparent. In most parasitic infections the incubation period is similar to the prepatent period, which is the time period before the parasite is detected in tissue samples or excretions. The effector systems that are detectable in this phase in naïve subjects (characterized in that they have not previously been in contact with the pathogen) are termed innate immune mechanisms. These mechanisms have a broad spectrum of activity. For instance, many different bacterial species are killed by lysozyme, a molecule that is constitutively present in secretions of mammals. Similarly, the complement, kallikrein, and coagulation systems are present and activated as part of the acute phase response [13]. Some of the key molecules for such activation are Hageman factor and complement factor C3, and these can be activated by a number of pathogen-derived molecules, which leads to further development of the inflammatory response [14]. Aside from direct activation of enzyme systems in plasma, pathogen-derived molecules activate phagocytic cells that upon specific interaction of a pathogen recognition receptor (PRR) produces interleukins 1 and 6 and tumor necrosis factor (TNF) with pro-inflammatory effects. These cytokines trigger the release and production of acute phase proteins, such as CRP,which itself is able to coat the infectious agent and facilitate uptake by granulocytes, in a process called opsonization [13]. It may be clear that a proportion of the pathogens is destroyed in the early phase of infection.

Onset of Adaptive Immune Response

Depending on the efficacy of the innate immune responses, a number of pathogens survive for a longer period of time in the infected host. This continues until the additional effector mechanisms of the adaptive immune response become effective, thus tilting the balance in favor of the host. The onset of the adaptive immune response is immediate after infection, with pathogen-derived antigens being taken up and processed by antigen-presenting cells (Figure 10.1). The antigen-presenting cell travels from the periphery to specific areas in the secondary lymphoid organs where lymphocytes are located. Antigen-presenting cells are crucial in the adaptive immune response because they present the antigen at the cell surface and bring it into contact with specific antigen receptors on functional lymphocytes (signal 1; [15]). A number of nucleated cells have the capacity to take up antigen and present antigen-derived epitopes at the cellular surface. Most of them are leukocytes, but under the influence of certain cytokines, other cells than leukocytes such as endothelial cells also perform this function. The cell surface receptors involved in this uptake are pathogen recognition receptors and belong to different families of receptors. The

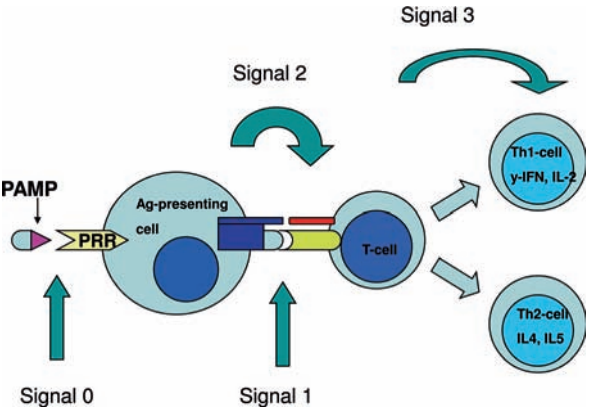

Figure 10.1 Regulation of the adaptive immune response. Pathogens or molecules derived from them exhibit pathogen associated molecular patterns (PAMP) that are recognized by germ line-encoded pathogen recognition receptors (PRRs, e.g., Toll-like receptors, signal 0). The antigen is internalized and immunogenic epitopes are presented to lymphocytes (signal 1). Co-stimulation of the antigen presenting cell generates a second signal that is required for T-cell activation (signal 2). Whether activated T-cells differentiate to Th-1- or Th-2-cells depends upon a specific cytokine context, referred to as signal 3.

Toll-like receptors (TLR) are membrane-bound receptors, whereas the nucleotide-binding oligomerization domain (NOD) receptors are cytoplasmic [16, 17]. These receptors signal to the nucleus and regulate the transcription of genes that encode for proteins with regulatory functions (e.g., cytokines). The close contact between antigen-presenting cell and functional lymphocyte (called immunological synaps) allows cytokines to exert their functions in the microenvironment of the cells (signal 2).

Outcome: Recovery or Chronic Infection/Death

Upon stimulation of the antigen receptor and co-stimulation of additional receptors, lymphocytes are activated and develop into effector cells. There are essentially two types of lymphocytes defined by the primary lymphoid organ where they have matured: T-lymphocytes and B-lymphocytes. T-lymphocytes produce cytokines and may be cytotoxic. B-lymphocytes develop into antibody-forming plasma cells. Cytokines and antibodies may help to control the proliferation of the pathogen, leading to elimination and cure of the infected subject. In some cases the adaptive and innate responses are inadequate, which can lead to chronic infection and ultimately death. This may be related to the type of immune response that is triggered: T-helper 1 cells (Th-1) response or T-helper 2 cells (Th-2) response [18]. Immune responses that are dominated by Th-1 are characterized by the production of the cytokines gamma-interferon (γ-IFN) and interleukin 2 (IL-2), whereas responses dominated by Th-2 are characterized by the production of IL-4 and IL-5 [18]. The balance between Th-1 and Th-2 depends on the cytokine context in the microenvironment in which the antigen-

presenting cell plays a crucial role (referred to as signal 3; see above [19]). The importance of such balance is best exemplified in the *Leishmania major*–mouse model: mice that develop a Th-1 response characterized by γ-IFN production are immune to challenge infection, whereas mice that develop a Th-2 response characterized by antibody production and absence of γ-IFN production succumb upon challenge infection [20].

Control of Infectious Diseases

For many years the idea has been that it would be best to eradicate the pathogen. This has been accomplished with smallpox vaccination, but the reality is that this is an exception to the rule. Aside from theoretical considerations that it might be better to live in symbiosis with the pathogen than to eradicate it, the fact is that it is virtually impossible to target all possible hosts of the pathogen and/or the niches they inhabit. The best example is the malaria eradication program that was carried out in Sri Lanka in the 1960s, using DDT to kill mosquitoes and chloroquine to combat the malaria parasite. Although the incidence of malaria dropped to almost zero, within 10 years after eradication the malaria situation had reverted to pre-eradication levels [21]. Experiences like these led to the conclusion that our aim should be to control the disease rather than to eradicate the pathogen; the latter approach even bears the risk that drug-resistant parasites and vectors could emerge. There are many measures that can be taken to control infectious disease, the first being to avoid contact with the pathogen through elementary hygiene measures, such as sanitation [22]. In the case of vector-transmitted diseases the use of repellents and (impregnated) bed nets add to diminishing the risk of contracting the infection. Drugs and vaccines are used routinely as prophylactic measures; in many instances these complement each other (Table 10.1). Presently there is a tendency not to choose for either a drug or a vaccine but to use both tools as part of a comprehensive control program [23]. How these tools may interact and allow innate and adaptive immune mechanisms to control the infection is exemplified below. The majority of the examples presented below are taken from the field of parasitology, but the same principles may apply to other infectious diseases.

Chemotherapeutic Treatment

In situations where no vaccination protocol exists, drugs may be used to prevent clinical infection, and ideally, sustain the development of protective immunity. The best known example in human medicine is malaria prophylaxis [21]. A number of prophylactic drugs are available and used by subjects who temporarily visit malaria endemic areas. Because this treatment prevents establishment of infection there is no development of immunity. In contrast, the autochthonous population relies on the

chemotherapeutic cure of malaria infection, which in many cases is not effective or is too late, still causing 1–2 million deaths/year. After a number of malarious periods solid immunity develops [21].

In animal health prophylactic treatment of dogs with imidocarbdipropionate, a chemotherapeutic drug to cure *Babesia* infection, was evidenced but never gained much practice [24]. In contrast, drugs are used extensively in broiler production. Because of the high stocking density, broiler flocks are vulnerable to outbreaks of clinical coccidiosis caused by *Eimeria* parasites. This risk is managed with coccidiostatic drugs that are normally used in either rotation or shuttle programs to minimize the risk of selection for drug-resistant *Eimeria* parasites [25]. Usually flock immunity may develop, but there is great heterogeneity among the individual birds.

The same accounts for the control of helminth infections. Most of these drugs are safe, cheap, and effective against a broad spectrum of parasites. However, there is an increased incidence of emerging parasites that are resistant to the commonly used drugs [23] (see Chapter 2).

Chemotherapeutically Controlled Infection

Instead of waiting for a natural infection to occur and subsequent chemotherapeutic treatment with the objective to develop solid immunity, it was realized that deliberate infection and subsequent cure would present a more pragmatic and feasible approach. This is being practiced in a number of infectious parasitic diseases, especially blood parasites such as *Cowdria* (now called *Ehrlichia*) and *Theileria* species [26, 27]. The parasite strains used for such treatment are usually virulent when given without chemotherapeutic drug. The drug of choice is presently a long-acting tetracycline.

Although not intended as such, immunity against *Eimeria* infection in broiler flocks may result from chemotherapeutically controlled infection, especially when ionophoric drugs are used as a prophylactic control measure. Initially when ionophoric drugs were screened for coccidiostatic activity in chicken that were housed in wired cages, they were considered mediocre [28]. However, when chicken were given the same regimen but were housed in floor pens which increased accidental re-infection with shed oocysts, the coccidiostatic effects were much more dramatic. It appeared that the low number of oocysts that survived despite the ionophore treatment evoked solid immunity in the flock, which added to the reduction of parasite proliferation. This principle has later been exploited commercially (see below).

Low-Dose Infection

The ancient practices of variolation and leishmanization are examples of vaccination by low-dose infection, although at the time this was not realized. To date leishmanization is still practiced (inoculation at the buttocks), mainly to avoid the develop-

ment of the classical oriental sore due to natural infection. After healing, vaccinated subjects have a robust cellular immune response, revealed by positive lymphocyte stimulation test [29].

In some cases low-dose infections ensue although the strain used is regarded virulent in other situations. For instance, calves are less susceptible to *Babesia bovis* infections as compared to adults, possibly due to the presence of a babesiacidal factor in the serum [30]. The current practice is to vaccinate calves with *B. bovis* and *B. bigemina* parasites in the first six months of life. Maternal immunity is a compromising factor that may help an immunizing infection to develop a mild course. In contrast, strong maternal immunity may interfere with the induction of immunity [31].

The problem with the development of flock immunity against coccidiosis in chickens, as described above, resides in the fact that the time period before a bird comes into contact with the pathogens is different among the flock members. Chickens that become infected with a low number of oocysts that contaminate the broiler house will expand this number 10 000–100 000-fold within one week, thus increasing the risk that naïve flockmates receive a high-dose infection with resulting clinical disease. This can be overcome by infecting the chickens all in the same day, usually the day of placing in the broiler house. Generally, chickens are immune after one or two subsequent infections that booster immunity [32].

Cross-Species Vaccination

The original finding of Jenner that inoculation with cowpox induced immunity against smallpox is probably the first example of cross-immunity. In chickens vaccination against Marek's disease virus is done with herpes virus of turkey (HVT) [33]. Similar approaches have been used in parasitology: in cattle *Anaplasma centrale* has been used to induce protection against challenge with *A. marginale* infection [34]. This effect is different from the refractory state induced in mammals upon (over-)stimulation of the reticuloendothelial system (RES) with bacterial suspensions such as *Corynebacterium parvum*. In these cases subjects are insensitive to infection for a relatively short period of time after treatment, but this state lacks immunological specificity and memory [35].

Infection with Attenuated Strains

Attenuation is the strategy/process to obtain a less virulent variant of a pathogen. There is no single technique being used; in contrast a plethora of methods are employed to obtain less virulent vaccine strains varying from repeated passage *in vivo* or *in vitro*, treatment with chemicals or gamma irradiation. As this also comprises serial passages in other than the target animal (for instance the *Toxoplasma gondii* S48 vaccine, see below) it can be argued to what extent cross-species vaccination differs from that using attenuated strains. When using passage techniques it is not known

what the genetic basis for attenuation is, hence it is not clear whether this change is stable or that it will be repaired. For that reason reversion to virulence studies are needed such as back-passage of the attenuated vaccine strain through the target animal under nonrestrictive conditions [36]. It needs to be shown that the attenuation is stable for at least five back-passages or for the number of passages that is used for the production of vaccine. In cases it is not feasible to passage the pathogen *in vitro*, or when attenuation is not achieved with such procedure, it may be possible to grow the *virulent* organism but to attenuate it by a subsequent treatment. Gamma irradiation has been successfully used to attenuate lungworm parasites [37]. When the infectious L3 larvae are harvested and subsequently irradiated, they lose the capacity to complete development beyond the L4 and mature lungworm stages, whilst allowing protective immunity to develop.

With the advent of molecular biological techniques, an entire new array of possibilities was introduced. Genetically modified *Leishmania mexicana* parasites from which a virulence gene was knocked-out were constructed and could be used successfully to immunize mice [38]. In *Toxoplasma gondii* a "drug addiction gene" was introduced that allowed the propagation of the vaccine strain in the presence of the drug but prevented the vaccine strain from further proliferation in the mammalian host, whilst inducing protective immunity [39]. There are clear opportunities for this kind of methodology.

Non-Live Vaccines

Safety of vaccines is paramount, which is the reason that initial attempts are to grow the pathogen and subsequently inactivate it. Feasibility studies are then performed to determine the immunizing potential of such preparations. Killed bacteria (called bacterins) may be successfully used as vaccine as such without an adjuvant, due to the presence of lipopolysaccharide, techoic acid, and other cell wall constituents [40], but most of the inactivated vaccines require an adjuvant to provide the necessary signal 2. Chemical inactivation is the most frequently used. Treatment with formaldehyde, beta-propiolactone (BPL), and bromoethylamine (BEA) are commonly used. Other methods of inactivation are ultrasonic treatment, heating, UV irradiation, and gamma irradiation. There are a few examples of parasitic vaccines that are produced along these principles: against *Neospora caninum*-induced abortion in cattle [41], against *Giardia lamblia* infection in dogs [42], and against *Babesia divergens* in Austria [43]. Each of these vaccines contains an adjuvant to stimulate the proper response.

Subunit Vaccines

A vaccine that does not contain whole organisms is called a subunit vaccine. In general, subunit vaccines contain (partially) purified antigens from whole, killed organisms. Purification may be necessary to remove putative toxic antigens that may compromise the safety of the vaccine, or to remove immunodominant antigens

that jeopardize the immunogenicity of the protective antigen. Subunit vaccines usually require more downstream processing, which adds to the cost price of the product. Because of this, subunit vaccines are mainly developed for companion animals. Recently a subunit vaccine against *Leishmania infantum* infection in dogs was developed. It contains purified fucose/mannose ligand from *L. infantum* and is formulated with muramyl di-peptide adjuvant [44]. Special forms of subunit vaccines are those that contain excreted/secreted antigens of the pathogen. Examples are the vaccines against *Babesia canis* and *B. rossi* in dogs [45, 46].

A special form of subunit vaccines are those that are produced by recombinant DNA technology. The isolated gene that encodes for an immunoprotective pathogen-encoded protein is inserted in an appropriate expression system. This technique allows (over-)production of the relevant antigen. The vaccine against the sheep tapeworm *Taenia ovis* is based on a recombinant-oncosphere antigen [47]. Vaccination provokes antibodies against this molecule, which interfere with the infective parasite stage (oncosphere) attaching to the gut wall. On the same principle, vaccines against other tapeworms have been developed [47]. Another example is the recombinant-merozoite antigen (Bd37) of *Babesia divergens* [48]. It was shown that both active vaccination with the recombinant antigen and passive vaccination with the monoclonal antibody directed against Bd37 provided protection against challenge infection (Kleuskens, Crommert, Janssen, Précigout, Carcy, Gorenflot, and Schetters, unpublished data).

DNA Vaccines

Vaccines that do not contain the protective antigen but contain the DNA that encodes the vaccine antigen are called DNA vaccines [49]. These can be live viral vectors but also non-live DNA fragments that are injected into the host under circumstances that maximize the chance that the DNA enters a nucleated cell. In addition, the feasibility of messenger RNA to induce protective immunity has been evidenced [50]. These approaches seem very promising in the field of virology but in bacteriology and parasitology such vaccines are not available yet. Improved responses can be obtained when subjects are primed with a DNA vaccine and boosted with the (recombinant) protein or viral vectors encoding the vaccine antigen [51, 52]. Although this holds promise, such combinations are presently not commercially feasible.

Therapeutic Vaccination

Similar to the therapeutic use of drugs for infected subjects, there is the possibility to use vaccines as therapeutics. The earliest example of therapeutic vaccination is the boy that was bitten by a rabid dog and treated with an attenuated rabies vaccine developed by Louis Pasteur in 1885 [53]. With the possible threat of biological warfare using smallpox (after smallpox eradication, vaccination was relaxed), rapid vaccination after contact with smallpox has been proposed [54]. Therapeutic vaccination is

also used in the treatment of HIV-infected patients in combination with antiviral therapy, with mixed results. In *Leishmania* infections in dogs, it was shown that vaccination of parasite-positive dogs reduced the parasite burden. When combined with a chemotherapeutic drug the effect was greater [55]. Also in the field of bacteriology, therapeutic vaccination is being pursued as illustrated by the post-exposure vaccination therapy to treat latent *Mycobacterium tuberculosis* infection [56]. Therapeutic vaccination is called for to cure nonclinical cases of *M. tuberculosis* infection, since such cases pose a potential threat when the infection is re-activated. For ovine Jones Disease in sheep (caused by *M. paratuberculosis*) a vaccine with therapeutic properties is commercially available [57].

Conclusions

Antiparasitic drugs are an important tool to prevent and control parasitic infections. In the light of the development of drug resistance and the selection of resistant strains, this use of drugs is more rationalized and impetus is given to research into the development of vaccines. Basic vaccine research is directed at manipulating the innate and adaptive immune system in order to trigger the immunoprotective response. Key to this are the vaccine adjuvants and immunopotentiators that orchestrate the immune response through signal 1, 2, and 3 (Figure 10.1) [58]. It is envisaged that these new tools will be used strategically to control infectious diseases, which means that, in addition to management practices that limit the level of contamination, the alternate and/or combined use of drugs and vaccines will be practiced.

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Part Two
Protozoan Parasites

11
Proteases of Parasitic Protozoa – Current Status and Validation

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Abstract

Proteases are successfully targeted in many human pathophysiologic and infectious diseases, exemplified by drugs against hypertension, osteoporosis, type II diabetes, and HIV/AIDS. Proteases that are indispensable or that function at critical bottlenecks in the parasite lifecycle are sought after, and several from parasitic protozoa impacting public health and animal welfare are currently being studied. Malaria alone infects 300–500 million people and causes approximately 3000 deaths daily, most of which represent children less than five years of age in sub-Saharan Africa. It is estimated that a total of 500 million humans are exposed to *Trypanosoma brucei*, *Leishmania*, and *Trypansoma cruzi*, the causative agents, respectively, of African sleeping sickness, leishmaniasis, and Chagas' disease. Advances in genetic and selective chemical targeting of proteases in parasitic protozoa have shed light on the discrete biological roles that these enzymes play, and serve as a necessary tool in the target validation process. This chapter highlights the current status in the target validation process of all classes of proteases from parasitic protozoa of human clinical and veterinary significance.

Plasmodium

Plasmodium spp., the unicellular parasite that causes malaria, is transmitted following the bite of an infected anopheline mosquito. Sporozoites migrate to hepatocytes where the parasite replicates to produce several thousand merozoites per cell. Following rupture the merozoites enter the bloodstream and invade erythrocytes. This initiates the erythrocytic cycle that is responsible for the clinical manifestations of the disease. At each round of subsequent intra-erythrocytic growth, the parasite divides asexually

inside a parasitophorous vacuole (PV), digesting the host cell hemoglobin and producing 16–32 daughter merozoites/cell, which subsequently egress to invade fresh erythrocytes and repeat the cycle.

Approximately two-thirds of the world's population are exposed to malaria but there is no licensed malaria vaccine, so prevention and treatment of the disease relies primarily on vector control and antimalarial drugs. The most dangerous form of human malaria is caused by *Plasmodium falciparum*, and resistance of both this species and *P. vivax* – which probably causes many more cases of clinical malaria worldwide – to many previously invaluable antimalarial drugs such as chloroquine and several antifolates is widespread. In response to this continuing drug crisis, the exploration of new drugs and new drug targets such as proteolytic enzymes is a priority. The evidence to date indicates two main elements of *Plasmodium* biology that might be targeted by protease inhibitors: hemoglobin digestion and host cell entry/exit. The strategies being explored to investigate these are described below.

Function and Essentiality of the Hemoglobinases

As the intraerythrocytic asexual blood-stage parasite develops, host cell hemoglobin is endocytosed via a specialized apparatus called a cytostome, delivered to an acidic, lysosome-like digestive vacuole (DV) and degraded. To date, four parasite aspartic endopeptidases – plasmepsins I, II, III (otherwise known as histoaspartic protease, or HAP), and IV – have been implicated in hemoglobin digestion [1, 2]. In addition, at least three papain-like (clan CA) cysteine endopeptidases called falcipain 2, 2′, and 3 are thought to play a role in the process [3–5]. Degradation of the resulting peptides to shorter peptides and eventually to amino acids is thought to be mediated by a DV-located metalloprotease of the M16 family called falcilysin [6, 7], plus at least one dipeptidyl aminopeptidase called DPAP1, with the additional involvement of several nonmethionine aminopeptidases, all members of the metallopeptidase family [8–13]. To add to this complexity, some of the enzymes appear to have additional roles distinct from hemoglobin digestion; for example, very recent evidence implicated falcipains in activation of plasmepsins following their delivery to the DV [14], falcilysin may be involved in the functioning of the apicoplast, a chloroplast-like malarial organelle [7, 15, 16], and falcipain-3 may be involved in sexual differentiation of the parasite in the mosquito vector [17].

Potential of the Hemoglobinases as Drug Targets

The available evidence therefore strongly suggests that – in isolation – pharmacological inhibition of any of the individual aspartic hemoglobinases plasmepsin I, II, IV, or HAP, or falcipains 2 and 2′, would be unlikely to efficiently control parasite replication *in vivo*. However, there is still potential for drugs targeting falcipain-3, or combinations targeting both the cysteine and aspartic proteases, or alternatively drugs designed to simultaneously inactivate all the members of each mechanistic

class. Several of these proteases have been expressed in recombinant form, enabling the development of high-throughput screens to aid the drug discovery effort, and substantial efforts are still being directed towards the development of antihemoglobinase drugs; as an example of this, the search for drugs targeting falcipains remains a high-profile project in the drug discovery portfolio of the Medicines for Malaria Venture (www.mmv.org), a nonprofit public–private partnership devoted to the development of new antimalarials. Both falcilysin [15] and DPAP1 [18] appear to be essential genes, and so will undoubtedly remain of interest to groups focused on hemoglobinases as therapeutic targets.

Proteases Involved in Host Cell Exit and Entry

Both egress of blood-stage parasites and subsequent merozoite entry into the new erythrocyte involves the action of parasite proteases. Early investigations showed that schizont rupture could be efficiently blocked by the presence of certain broad-spectrum protease inhibitors. The demonstration therefore that disruption of the gene encoding a putative papain-like protease called SERA8 results in a complete block of sporozoites release from oocysts in the mosquito vector [19] was an important breakthrough. SERA8 belongs to a family of nine similar papain-like proteins encoded by the *P. falciparum* genome. Gene disruption studies have indicated that while some are dispensable, SERA5 and 6 – which both localize to the PV – are essential in asexual blood stages [20, 21]. In the most recent demonstration of this, processing of SERA4, 5, and 6 was shown to be mediated by an essential subtilisin-like serine protease called SUB1, which is released into the PV space from an unusual set of dense granule-like organelles just prior to egress [22]. Inhibition of PfSUB1 function resulted in a block in egress. Implicit in these findings (although not yet proven) is that SERA processing by PfSUB1 represents the activation of these proteases, and that this in some manner leads to egress. Clearly, many questions remain unresolved on this issue, not least of which is precisely how proteases can mediate destabilization of the PVM and erythrocyte membranes.

Numerous studies have implicated protease involvement in erythrocyte invasion, and most of this evidence points to a primary role for serine proteases (reviewed by [23]). The identity of these enzymes has only relatively recently become clear, with the available data pointing to the involvement of two distinct serine protease activities in invasion. One of these is a membrane-bound subtilisin-like protease called SUB2 [24, 25] that is responsible for the shedding of at least one abundant glycolipid-anchored merozoite surface protein complex at invasion [26]. SUB2 also sheds two micronemederived type I integral membrane proteins called AMA1 and PTRAMP [26–28]. The second serine protease involved in invasion, ROM4, belongs to the structurally quite distinct rhomboid intramembrane protease family. ROM4 seems to have a distinct role from SUB2, since its substrates in asexual blood stages cannot include glycolipid-anchored proteins and may even be restricted to a set of type I integral membrane erythrocyte-binding ligands called the DBL-EBP family, which includes the well characterized *P. falciparum* sialic acid-binding protein EBA-175, [29, 30].

Whilst most investigations of proteases in invasion are centered on merozoite invasion or erythrocytes, there is recent evidence suggesting that hepatocyte invasion by the sporozoites also requires the activity of a parasite protease. In this case, an anonymous cysteine protease activity modifies the major sporozoite surface protein, CSP, to expose a host cell-binding domain [31, 32]. This finding raises clear possibilities for targeting early stages in malarial infection with protease inhibitor-based approaches.

Potential of Egress and Invasion-Related Proteases as Drug Targets

In principle, drug-mediated inhibition of egress should provide an effective route to disease control since the asexual blood-stage malaria parasite needs to migrate from one host cell to another to continue to replicate. Furthermore, although relatively little is known of egress of liver stage merozoites, all the evidence indicates that this shares many features at the molecular level with blood-stage schizont rupture, and there are indications that many of the molecular players are in common [33, 34]. As a result, targeting the proteases involved may provide a "double whammy," attacking two distinct stages in the parasite lifecycle. But do these proteases have potential as drug targets? Many questions remain over the function of the known essential members of the SERA family. In particular, the presence of a Ser residue in place of the canonical catalytic Cys in SERA5 leads to speculation over whether it is a protease at all. However, recombinant SERA5 has been demonstrated to possess low-level chymotrypsin-like activity [35], so in principle this sets the scene for a better understanding of its function and the identification of selective inhibitors. SUB1 is predicted to have good potential as a target; its closest homologs are bacterial subtilisins (thus quite distinct from known host enzymes), it is encoded by a single-copy, essential gene, it has already been shown to be druggable, and the availability of recombinant enzyme, fluorogenic substrates and an HTS should all contribute towards the identification of more potent, drug-like inhibitors suitable for preclinical evaluation [22].

Trypanosoma cruzi

Chagas' disease, caused by infection with the parasite *T. cruzi*, is the main cause of heart disease in Latin America. Infection leads to progressive cardiomyopathy and significant mortality of young adults, while gastrointestinal mega-syndromes are common in Brazil [36, 37]. Around 60% of acute cases occur in children. Reactivation of Chagas' disease leading to meningoencephalitis and/or acute myocarditis also occurs in immunosuppressed patients [38–40]. Treatment may decrease mortality in these patients but *T. cruzi* infection persists.

Cruzain (also known as cruzipain) is a validated drug target [41–43]. This abundant cathepsin L-like cysteine protease localizes to lysosomes, the cell surface, and

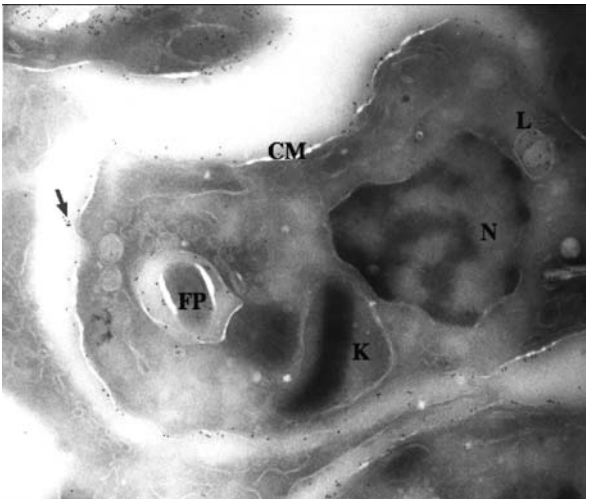

Figure 11.1 Immunoelectron microscopy localization of cruzain (arrow) in an intracellular T. cruzi amastigote within the cytoplasm of a skeletal muscle cell. CM, cell membrane; FP, flagellar pocket; K, kinetoplast; L, lysosome; N, nucleus (45 000×).

the flagellar pocket of pathogenic *T. cruzi* amastigotes (Figure 11.1) [42]. Cruzain has pro-, catalytic, and C-terminal domains that are processed as the protease transits through the ER and Golgi complex [42–46]. The first evidence of the trypanocidal mechanism of cysteine protease inhibitors came from ultrastructural studies that showed Golgi complex alterations while radiolabeled inhibitors provided target validation. Treatment with cysteine protease inhibitors induced cruzain accumulation in Golgi cisterns by preventing its normal autocatalytic processing and intracellular trafficking and resulted in parasite death [41, 42]. In contrast, inhibitors had no significant effect on mammalian cathepsins, presumably because of their redundancy and activation within other subcellular compartments.

A library of thousands of cysteine protease inhibitors with different scaffolds and chemical structures screened in enzymatic assays has provided several lead drugs (www.collaborativedrug.com). Fluoromethylketones, vinylsulfones, diazomethanes, bis-acridines, fluorogenic peptides containing phenylalanine, and thiosemicarbazones inhibit cruzain activity [47]. The lead drug, K11777, was identified and has a good safety profile, PK/PD properties, and oral bioavailability, and is a good drug candidate for diseases as diverse as trypanosomiasis and schistosomiasis [47].

Trichomonas vaginalis

Trichomonas vaginalis is a sexually transmitted human pathogen responsible for more than eight million cases of trichomoniasis each year in the United States, and over

80–100 million cases worldwide annually [48]. *T. vaginalis* is also the most common nonviral sexually transmitted disease (STD). Correlations of infection with *T. vaginalis* and increased infections with HIV and other sexually transmitted diseases have highlighted the importance of this human parasite in recent years. Cysteine proteases have been the focus of drug discovery and development against other protozoan and helminth parasites; and their molecular and biochemical distinction from host cysteine proteases and the elucidation of basic biological functions of cysteine proteases in trichomonads have emerged as a new area of study. Our knowledge of the biochemistry and molecular biology of cysteine proteases from *T. vaginalis* is extremely limited. Several mechanisms of *T. vaginalis* pathogenesis have been proposed, some of which strongly suggest an involvement of cysteine proteases. In *T. vaginalis*, cysteine proteases are implicated in the degradation of the extracellular matrix of the host cell and destruction of host immunoglobulins, and even the destruction of host protease inhibitors [49]. To date, several cDNA clones encoding putative cysteine proteases (denoted as CP1, 2, 3, etc.) have been isolated and characterized to some extent [50], although the *T. vaginalis* genome project revealed a number of annotated sequences encoding putative cysteine proteases [50] all of which showed strong homology to the human cysteine protease cathepsin L. Expression of the four genes corresponding to these cDNAs could be detected using RNA dot blot analysis, and Southern blot analysis revealed differences in gene-copy numbers for these protease genes.

Using two-dimensional and gelatin gel electrophoresis, several protease spots of differing molecular weights have also been detected [51]. One spot in particular (~65 kDa in size, named CP65) was analyzed using a variety of cysteine protease inhibitors. Antiserum raised against these excised E-64-sensitive protein spots was used in immunofluorescence studies to localize the putative protein to the cell surface and the cytoplasm; the antiserum was also able to reduce the level of *T. vaginalis*-associated cytotoxicity to HeLa cells. Like CP65, the gene and protein sequences are still lacking.

Recently [43], isolated *T. vaginalis* CP2, CP3, and CP4 from the extracellular milieu of cultured parasites associated with human vaginal epithelial cells and confirmed their identities using peptide sequencing of the purified proteases. The irreversible cysteine protease inhibitor E64 was shown to have little effect on the *in vitro* growth of parasites, suggesting that many of these enzymes are not essential for growth in axenic culture [52]. However, Sommer [53] observed inhibition of host cell apoptosis when cysteine protease enriched fractions were pre-treated with the inhibitor E-64, and Sommer and colleagues [53] recently demonstrated a direct role for cysteine proteases in inducing apoptosis in human vaginal epithelial cells, strongly implicating these enzymes in the pathogenesis of *T. vaginalis*.

In another study, León-Félix and colleagues [54] identified 23 distinct protein spots with proteolytic activity on two-dimensional (2-D) gel electrophoresis. The six proteinases correspond to two distinct cysteine protease clans: Clan CA enzymes, represented by four spots and Clan CD enzymes represented by two spots. Arroyo and colleagues isolated two cDNAs encoding for legumain-like CPs from *T. vaginalis*, called *Tvlegu-1* and *Tvlegu-2*. The two sequences share approximately 40% identity at

the amino acid level. However, they do not correspond either to the typical asparaginyl endopeptidase or the glycosylphosphatidylinositol (GPI): protein transamidase subfamilies, and may represent a new family.

Babesia

The genus *Babesia* comprises of more than 100 species of protozoan intra-erythrocytic pathogens that infect a wide variety of vertebrate hosts [55]. They are transmitted by their tick vectors during the ingestion of a blood meal from the vertebrate host [55, 56]. Babesiosis is one of the most common infections of animals worldwide and is gaining increasing interest as an emerging zoonosis, which is a disease communicable from animals to humans. *Babesia bovis* is a cattle pathogen imposing a global risk and severe constraints to livestock health and economic development. Human babesiosis is caused by one of several babesial species that have distinct geographical distributions based on the presence of competent hosts. The spectrum of disease is broad, ranging from an apparently silent infection to a fulminant, malaria-like disease, resulting occasionally in death. When present, symptoms typically are nonspecific (fever, headache, myalgia) [57]. With no vaccine available for either veterinary or human disease, research into drug-mediated interference in the life cycle is sorely needed. Parasite proteases show the biggest potential as targets for such chemotherapy.

The invasion, growth, and maturation of *Babesia* within the erythrocyte is accompanied by many changes in both the parasite and the host red cell. Babesia-derived proteases are thought to be of particular importance in causing these modifications. As in *Plasmodium*, proteases in *Babesia* are thought to exert their effect at three different time points in the intra-erythrocytic cycle: (1) during invasion, (2) during hemoglobin hydrolysis,- and (3) at host cell rupture.

Proteases in *Babesia* have not garnered much research attention. Results using a wide variety of relatively broad-spectrum protease inhibitors on the invasion and growth of *B. divergens* in culture show that, of the different categories of inhibitors, those specific for serine proteases had the greatest impact on the invasion of *B. divergens* [58]. Treatment of *B. divergens* parasites with E64 or pepstatin (inhibitors of cysteine and aspartic protease, respectively), antipain (cysteine and serine proteases inhibitor) or PMSF and ABSF (serine protease inhibitors) had a moderate effect on the invasion and growth of *B. divergens* parasites into the red blood cells (RBCs). However, TPCK, TLCK, and 3, 4 DCI (irreversible serine protease inhibitors) severely decreased the invasion by free merozoites into the RBCs. TPCK and TLCK reduced invasion by more than 70%. Both serine protease inhibitors dramatically decreased the percentage of parasites that successfully invaded cells which was evidenced by a large number of free merozoites around the host RBCs, seen in Giemsa-stained smears (Figure 11.2). However, a similar study looking at the effect of cysteine protease inhibitors on growth in *B. bovis* found that only lipophilic inhibitors E64d and ALLN significantly inhibited parasite growth [59].

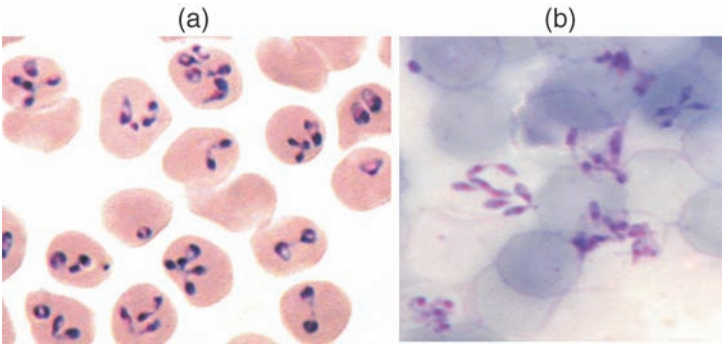

Figure 11.2 Protease inhibitors decreased successful RBC invasion of *B. divergens*. (a) Giemsa stained thin blood smears show results of normal invasion of parasites in the absence of inhibitors. (b) However, a high number of free extra-erythrocytic merozoites were visualized in the Giemsa smears of *B. divergens* cultures treated with protease inhibitor TLCK.

At the molecular level, studies have only recently begun to probe the identity of these targets of protease inhibitors. Using a functional proteomics tool, in the form of a biotinylated fluorophosphonate probe specifically directed against the active site of serine proteases [60, 61] the presence of two dominant serine proteases was demonstrated in asexual *B. divergens* [62]. One of these active serine proteases (p48) and its precursors were recognized by anti-PfSUB1 antibodies, in *B. divergens* crude lysates, leading to the cloning and characterization of the *B. divergens* subtilisin 1. BdSUB1 shows a significant homology to *P. falciparum* and *P. yoelii* subtilisins, with the highest degree of sequence identity around the four catalytic residues. Like subtilisin proteases in other Apicomplexa parasites, BdSUB-1 undergoes two steps of processing during activation in the secretory pathway being finally converted to an active form (p48). The mature protease is concentrated in the dense granules of free merozoites which are apical secretory organelles involve in erythrocyte invasion. Antibodies against BdSUB1 resulted in very similar phenotypes that were observed in the presence of TPCK and TLCK, namely low invasion efficiency and large numbers of free merozoites present in the culture medium [58]. Thus, this data lends further support to the pivotal role of serine proteases in *B. divergens* invasion and offers hope for the development of suitable anti-parasitic agents.

Entamoeba

Entamoeba histolytica is a protozoan parasite that causes amebic colitis and liver abscesses. Amebiasis is the second leading cause of death from parasitic diseases, resulting in an estimated 40 000–100 000 deaths/year, mainly in developing countries [63]. Cysteine proteinases are key virulence factors of *E. histolytica* and play a

central role in tissue invasion and disruption of host defenses. Although morphologically identical, invasive *E. histolytica* trophozoites secrete 10- to 1000-fold more cysteine proteinases than noninvasive *E. dispar* [64]. Cysteine proteinases purified from axenized *E. histolytica* (adapted to grow in sterile culture) cleave collagen, elastin, fibrinogen, and laminin [65], elements of the extracellular matrix that trophozoites must penetrate to cause invasive disease. Cysteine proteinases purified from *E. histolytica* can also specifically cleave the third component of complement [66], degrade IgA [67], IgG [68], and the anaphylatoxins C3a and C5a [69], which may explain the relative paucity of neutrophils noted in amebic liver abscesses. Active, recombinant EhCP5 also degrades pro- and mature IL-18, limiting the host response [70]. The role of cysteine proteinase in invasion has [71] also been confirmed in animal models.

Genome-wide homology searches found at least 50 genes of *E. histolytica* encoding cysteine proteases [72], most of which belong to papain superfamily. The majority encode homologous enzymes with a cathepsin L-like structure [73]. Among the predicted *E. histolytica* cysteine proteinases, some have putative transmembrane or glycosylphosphatidylinositol anchor attachment domains, suggesting they are membrane-associated proteases [74]. A few of them are similar to calpain-like cysteine proteinases, ubiquitinyl hydrolase, Ulp1 peptidase, autophagin, and otubain [75]. The expression of cysteine proteinase genes in cultured trophozoites differs from those *in vivo*. Only seven cysteine proteinase genes were detected in cultured amebae, and only three EhCP1, EhCP2, and EhCP5, at high levels [74, 75]. During cecal infection *in vivo*, different cysteine proteinase genes were upregulated, including EhCP4, EhCP6, EhCP9 [76], suggesting that expression of *E. histolytica* cysteine proteinases is dependent upon physiological context, such as contact with substrates.

The recombinant enzymes characterized to date are EhCP1 [77], EhCP2, EhCP3 [11], and EhCP5 [78]; all have similar substrate specificity, requiring arginine in the P2 position, as predicted by homology modeling [73]. In addition, they all can cleave a broad range of fundamental physiologic substrates, such as mucin [79], extracellular matrix proteins [80], inflammatory cytokines [70], and IgG [68].

The biological properties of EhCPs and their important roles in *E. histolytica* pathogenicity make them attractive targets for drug development. Most studies have focused on the two proteinases specific to *E. histolytica*, EhCP1 and EhCP5. Antisense expressed to EhCP5 decreased liver abscesses in SCID mice [81] and hamsters [82]. The epoxide cysteine proteinase inhibitor, E-64, blocked amebic liver infection in SCID mice when preincubated with trophozoites [81], but it is too toxic to be used therapeutically. Vinyl sulfone-based inhibitors that modify the thiolate of the catalytic cysteine of cysteine proteases and irreversibly inactivate the enzymes look to be very promising parasitic cysteine protease inhibitors [83, 84]. K11777 is a water-soluble, orally bioavailable, nonmutagenic, potent inhibitor that is active against parasitic cysteine proteases with low toxicity. The compound has undergone extensive toxicity testing [85] and is approaching clinical trials for Chagas' disease. The efficacy of K11777 inhibitor was first tested in amebic colitis using a human intestine xenograft model [77] The K_{ass} (k_{inact}/Ki_{app}) of K11777 against recombinant EhCP1 is 350 (1/M·1/s). When *E. histolytica* trophozoites are preincubated with K11777, no invasion was identified in the colon xenografts by histology within 24 hours post-infection and real-time PCR assays

(sensitive to 10 trophozoites per xenograft) demonstrated a >80% reduction in the number of invading *E. histolytica* trophozoite. A more specific inhibitor was synthesized by substituting an arginine for phenylalanine in the P2 position of K11777 based on the distinct specificity for arginine in P2 by EhCP1. The new compound, WRR483, has a much better inhibition potency ($K_{ass} = 849\,000$ 1/M·1/s) against recombinant EhCP1 and inhibited amebic invasion in human xenografts >95% [77].

Toxoplasma

Almost one-third of the population of the United States and up to 90% in other areas of the world, are infected with *Toxoplasma* [86]. Infection can be acquired congenitally or by ingestion of pseudocysts in undercooked meat or oocysts in contaminated food or water. Acute infection may be asymptomatic or present as a mononucleosis-like syndrome. In immunocompetent patients, the replicating tachyzoites convert to quiescent bradyzoites, leading to latent infection for the rest of the patient's life. Primary infection during pregnancy may cause severe congenital defects and is a major cause of visual impairment later in life [87].

Cysteine proteinases of *T. gondii* have been shown to play important roles in host cell invasion and nutrient acquisition. The *Toxoplasma* genome project revealed that the redundancy of cysteine proteinase genes is significantly less in *T. gondii* than in most parasites [88]. *T. gondii* encodes only a single cathepsin B [89], a single cathepsin L (Huang *et al.*, unpublished data), and three cathepsin Cs [85].

Cathepsin B (TgCPB or Toxopain-1) localizes to the rhopteries [89], the only acidic organelle [90]. Inhibitors to TgCPB were identified by screening with active, recombinant protease [89]. A cell permeable peptidyl ketone inhibitor blocked the *in vitro* invasion of fibroblasts and their destruction [89]. Chemical inhibition of TgCPB also caused distorted rhoptry formation, suggesting that it might interfere with organelle biogenesis [89]. Knockout of TgCPB proved to be lethal, but an antisense construct could reduce expression by >60% [91]. A similar effect on *in vitro* invasion and multiplication was seen, and the TgCPB knockdown resulted in decreased invasion in a chick model of disseminated toxoplasmosis [91], validating TgCPB as a viable drug target. Vinyl sulfone-based inhibitors have been identified as potent, nontoxic cysteine proteinase inhibitors. The most developed is K11777 [85], which is effective against *Toxoplasma* invasion, intracellular multiplication, and dissemination in the chick model [92]. Further efforts will focus on developing *T. gondii* cathepsin B-specific inhibitors, which are effective in the low nanomolar range with at least 10- to 50-fold selectivity for the parasite versus human cathepsin B.

The cathepsins Cs of *T. gondii* are also potential drug targets and are encoded by three genes, of which only two are expressed in tachyzoites [93]. Cathepsin Cs are dipeptidyl peptidases, unlike the other members of the papain family of cysteine proteinase which have endopeptidase activity [94]. They are attractive drug targets based on the fact that TgCPC1 is the most highly expressed cathepsin in tachyzoites [93]. Because of

the redundancy of cathepsins in higher eukaryotes, knockout of cathepsin C in mice does not cause any histological abnormality, and T-cell activation defects could only be demonstrated *in vitro*.

Like *P. falciparum*, the cathepsin C of *T. gondii* (TgCP1) is secreted in dense granules and is important in breaking down peptides [18, 93]. The genes contain a tyrosine-based motif that is shown to result in trafficking of endosomal or lysosomal proteins in higher eukaryotes [89]. Degradation of peptides in the parasitophorous vacuole of *T. gondii* can be inhibited by the dimethyl ketone inhibitor, Gly-Phe-dimethyl ketone which was developed to inhibit human cathepsin Cs. Specific inhibitors to the parasite enzyme are needed as the IC_{50} to TgCP1 was high at 31 mM, and the methyl ketone inhibitors have unacceptable toxicity.

Several serine proteinases may prove to be drug candidates in the future. Several subtilisin-like serine proteinases have been identified in *T. gondii* and play a role in processing of key parasite proteins [95–97]. TgSUB1 has a GPI anchor and localizes to micronemes, where it is likely to be involved in processing of microneme proteins involved in motility [97]. A second subtilisin-like proteinase, TgSUB2, localizes to the rhoptries and processes the rhoptry protein, ROP1 [96]. Rhomboid proteins, intramembrane serine proteinases, have been shown to cleave surface adhesins required for invasion [98, 99]. These serine proteinases are promising drug targets, but drug design has been limited by the difficulty of expressing recombinant enzymes.

Cryptosporidium

C. parvum is found worldwide in lakes, ponds and other surface waters [100, 101]. In a recent study, up to 52% of British Columbia's "pristine" watersheds were contaminated with oocysts [101]. Even without bioterrorist assistance, *Cryptosporidium* recently emerged as a major threat to water supplies in developed countries because of its chlorine resistance, small size, and capacity to survive in the gastrointestinal and respiratory systems of fish, birds, reptiles, and >80 mammalian species [102–104]). *Cryptosporidium* is among the commonest enteric pathogens of domestic livestock. Infected sheep and cattle can shed immense numbers of oocysts into the environment (10^{10} per day) [104, 105]. In Quebec, 90% of dairy herds are infected with *Cryptosporidium*. In recent years, *C. parvum* has shown its potential to cause major waterborne epidemics in urban areas, the most spectacular of which was the massive Milwaukee outbreak in 1993 [106, 107]. Of the 800 000 individuals served by the contaminated South Milwaukee waterworks plant, it is estimated that ~403 000 were affected, an attack rate of 52% [107].

Infection with *C. parvum* occurs when oocysts are ingested in contaminated water or food [106]. Infection in early childhood can result in permanent growth stunting [108]. *C. parvum* can persist for months to years in immunocompromised individuals (e.g., AIDS) leading to wasting and death [106]. A critical feature of the life cycle that contributes to the severity of *C. parvum* infection in both immunologi-

cally competent and incompetent hosts is the capacity for merozoites and thin-walled oocysts to auto-infect, resulting in a rapid amplification of parasite numbers in the gut lumen [109]. Although the incidence of cryptosporidiosis has declined in wealthy countries with the widespread use of high-activity anti-retroviral therapy (HAART), it is estimated that up to 30–50% of HIV/AIDS patients in the developing world develop chronic cryptosporidial diarrhea [110]. The most effective agent at the current time is paromomycin, which provides only modest improvement in 20–30% of patients.

At the current time, relatively little is known about the role of proteases in the biology of *C. parvum* or the therapeutic potential of targeting such enzymes. In HIV-infected subjects, the addition of one or more protease inhibitors to HAART is credited with reducing the clinical incidence and importance of cryptosporidiosis [111, 112]. Forney and colleagues published a series of papers exploring the role of serine proteases in parasite excystation [113, 114] and the therapeutic potential of alpha-1 anti-trypsin in therapy alone and in combination with paromomycin [115, 116]. Nesterenko and colleagues described a metalo-dependent cysteine protease associated with the *C. parvum* sporozoite [117].

Among the apicomplexan parasites, proteases serve many purposes, including the facilitation of cell invasion, nutritive degradation of host proteins, and the modification of parasite proteins during life cycle transition. In particular, the Clan CA (papain) family of cysteine proteases (CP) has been identified as key enzymes for many protozoan parasites, including the Apicomplexa. Cryptopain is a known Clan CA protease identified in the *C. parvum* genome [103]. We tested K11777 over a range of concentrations (10–100 μM) using assay in five cell lines: CaCO2, MDCK, FHS, HCT8, and I 407. A dose response was observed in all cell lines with modest impact below 20 μM (100-fold reduction) but near complete inhibition of parasite growth at 40 μM and higher 1000- to 100 000-fold.

We tested K11777 at two doses and by two routes in the IFNγR-KO mouse model. The results of these experiments are summarized in Table 11.1. We challenged the animals with 500 sporulated oocysts and treated with 1 or 2 mg K11777 either by mouth (by lavage twice a day) or by intraperitoneal injection. Treatments began 48 h after challenge and continued for 14 days. The uninfected control animals remained healthy at all times while all of the infected animals became symptomatic at 6–8 days

Table 11.1 Treatment of *C. parvum* infected C57/BL6 IFNγR-KO mice with K11777.

Group #	1	2	3	4	5	6	7	8
Treatment	K11777	K11777	K11777	K11777	No Tx	DMSO	DMSO	K11777
Infection Status	Infected	Infected	Infected	Infected	Infected	Infected	Infected	Uninfected
Dose	1 mg bid	2 mg bid	1 mg bid	2 mg bid	N/A	Infected	Infected	Uninfected
Route	PO	PO	IP	IP	N/A	PO	IP	N/A
N	10	10	10	10	10	10	10	5
Survivors	9/10	6/10	4/10	5/10	0/10	0/10	0/10	5/5

bid: *bis in die* (twice daily); PO: *per os*; IP: intraperitoneal; Tx: treatment; DMSO: dimethylsulfoxide; N/A: not applicable; N: number.

after infection. These animals regained weight and appeared to be completely normal when they were euthanized. This is the only time that we have "saved" animals once they have become symptomatic with *C. parvum* infection.

African Trypanosomes

Over 60 million people in 36 sub-Saharan African countries are at risk of infection with human African trypanosomiasis (HAT) or sleeping sickness. It is estimated that between 50 000 and 300 000 new cases of HAT occur each year. HAT is a vector-borne parasitic disease that is transmitted by the bite of infected tsetse flies. The fatality rate in untreated patients is 100% and treatment is severely toxic. Two subspecies of the kinetoplastids protozoan are the etiological agents for HAT. *Trypanosoma brucei gambiense* occurs in west and central Africa and causes an acute form of the disease. The acute form of HAT in the southern and eastern regions of Africa is caused by *T. b. rhodesiense*. The disease caused by both species of *T. brucei* is fatal if left untreated. Only four drugs have been approved for the treatment of sleeping sickness in the past 90 years. One new category of targets recently identified in *T. brucei* is the Clan CA papain-like lysosomal cathepsin proteases. The preliminary success of K11777 and its ability to kill *T. cruzi* in animal studies, demonstrated the proof-of-principle that cathepsins could serve as valid chemotherapeutic targets in trypanosomatids [41]. A subsequent study with the diazomethane inhibitor Z-Phe-Ala-CHN_2 (which can inhibit both cathepsin L and cathepsin B-like proteases) demonstrated that the selective inhibition of cathepsins was also a viable strategy for killing *T. brucei in vitro* and in mice [118].

Only two genes (a cathepsin B-like and a cathepsin L-like) encoding cathepsins have been identified from the *T. brucei* genome project [119, 120]. The trypanopain gene encodes a cathepsin L-like protease. Its gene product encodes a polypeptide with 450 amino acids and an estimated molecular mass of 48 kDa [121]. Selective targeting of the trypanopain message by RNA interference is not lethal to *T. brucei*. However, in human blood–brain barrier model systems, *T. b. gambiense* loses its ability to cross the blood brain barrier after treatment with the cathepsin-L inhibitor K11777 [122]. This suggest that trypanopain may play a key role in allowing *T. brucei* to infect the brain and may still be a rational drug target.

The tbcatB gene encodes a polypeptide of 341 amino acids with a predicted molecular mass of 37 kDa. Selective interference of the tbcatB transcript by RNAi is lethal to *T. brucei* in culture. The phenotypes associated with interference of tbcatB message were similar to those observed when the parasite was treated with the cathepsin B inhibitor Z-Phe-Ala-CHN_2. These studies support the hypothesis that Z-Phe-Ala-CHN_2 kills *T. brucei* by inhibiting tbcatB. Since both trypanopain and tbcatB appear to function in lysosomes/endosomes of *T. brucei*, they are most likely important players for degrading macromolecules in these compartments. Transferrin is a host Fe-binding protein that is taken up by *T. brucei* via receptor-mediated endocytosis [123]. Once in the lysosome, transferrin is degraded by the lysosomal

cathepsins [124]. Since inhibition of this process appears to be an attractive strategy for new drug discovery, several small molecules have been synthesized and screened for their ability to specifically inhibit the lysosomal cathepsins of *T. brucei* and to inhibit the growth of trypanosomes grown *in vitro*. The thiosemicarbazones are probably the most notable small molecules having bioactivity against *T. brucei*. They have been effective at inhibiting lysosomal cathepsins and at killing the parasites without any signs of toxicity to mice in short-term studies [125, 126]. Most recently, a series of purine-derived nitriles representing the first reported inhibitors of TbcatB have been developed having submicromolar potency against TbcatB and *T. brucei*. Successes with these classes of small molecules supports the idea that tbcatB is a viable target for drug development against HAT [127].

Theileria

The disease theileriosis, caused by apicomplexan parasites within the genus *Theileria*, is a major constraint on ruminant production systems in tropical and subtropical regions of the "Old World." European cattle breeds are particularly susceptible and evidence has been reported that the parasite is extending its range within Europe [128]. In an effort to control the disease, a number of strategies have been developed, including vaccination and drug treatment [129]. None of the strategies employed are entirely satisfactory and emerging resistance to the most effective and widely used drug (buparvaquone) is suspected.

An intriguing aspect of *Theileria* biology is the ability of the most pathogenic species (*T. annulata*, *T. parva*) to manipulate the infected host cell, induce proliferation, and cause a lymphoproliferative disorder [130]. Detailed study has shown that parasite-mediated control includes elevated expression of bovine genes encoding matrix metalloproteases (MMPs) [131]. It is thought that expression of these genes is associated with virulence of the infected cell by promoting metastasis into organs of infected cattle, and it was demonstrated that migration of *Theileria*-infected cells can be reduced by a synthetic inhibitor of MMP activity [132]. Study of parasite-encoded peptidases is limited for *Theileria*, but it is likely they play a significant role in establishing infection within bovine cells and may provide novel targets for control. Despite the lack of experimental data, annotation of the *T. annulata* and *T. parva* genomes [133, 134] and incorporation of sequences into the MEROPS database [135] allows *in silico* analysis of *Theileria* peptidases to predict a putative biological role. In line with most organisms [92] around 2% (1.9% for *T. annulata*) of genes in the *Theileria* genome encode peptidases. To highlight peptidases that may function in establishment of the parasite (macroschizont stage) immortalized host cell and identify rational targets for inhibition, *T. annulata* peptidases were screened for possession of a signal peptide (for secretion across the parasite membrane), RNA expression pattern through the parasite lifecycle, and predicted location in the parasite-infected cell [136] (Table 11.2). Of the 80 putative peptidases predicted

Table 11.2 Putatively secreted *T. annulata* peptidases.

Family[a]	*T. annulata* (gene)[b]	*T. parva* (locus)[c]	Peptidase or homolog (subtype)[d]	MERNUM[e]	*T. annulata* annotation[f]	TMHMM[g]	GPI anchor[h]	Schizont EST hits[i]	Merozoite EST hits[j]	Piroplasm EST hits[k]	SignalP3.0[l]	Predicted location[m]
Pepsin family (A1)	TA02510	TP03_0056	Eimepsin	MER050489	Aspartyl protease precursor, putative	1	–	0	0	0	Signal peptide	Apicoplast
	TA05735	TP01_0802	Subfamily A1A unassigned peptidases	MER050474	Aspartyl protease, putative	0	–	0	0	0	Signal peptide	Apicoplast
	TA02750	TP01_0692	Subfamily A1A unassigned peptidases	MER053050	Pepsinogen, putative	0	–	0	1	0	Signal peptide	Apicoplast
	TA17685	TP03_0676	Family A1 unassigned peptidases	MER050506	Aspartyl (acid) protease, putative	0	–	0	0	0	Signal anchor	Apicoplast
Papain family (C1)	TA03720	No ortholog	Papain homolog (*Theileria*-type)	MER050490	Cysteine proteinase precursor	1	–	0	2	0	Signal anchor	–
	TA03725	TP03_0280	Papain homolog (*Theileria*-type)	MER050493	Cysteine proteinase precursor	1	–	0	5	8	Signal anchor	–
	TA03730	TP03_0281	Papain homolog (*Theileria*-type)	MER050495	Cysteine proteinase precursor	1	–	2	10	8	Signal anchor	–
	TA03735	TP03_0282	Papain homolog (*Theileria*-type)	MER050497	Cysteine protease precursor	1	–	0	5	18	Signal anchor	–
	TA03745	TP03_0284	Papain homolog (*Theileria*-type)	MER050498	Cysteine proteinase precursor	1	–	0	1	6	Signal anchor	–

(*Continued*)

Table 11.2 (*Continued*)

Family[a]	*T. annulata* (gene)[b]	*T. parva* (locus)[c]	Peptidase or homolog (subtype)[d]	MERNUM[e]	*T. annulata* annotation[f]	TMHMM[g]	GPI anchor[h]		Schizont EST hits[i]	Merozoite EST hits[j]	Piroplasm EST hits[k]	SignalP3.0[l]		Predicted location[m]
	TA03750	TP03_0285	Papain homolog (*Theileria*-type)	MER050500	Cysteine proteinase precursor		1	–	0	0	6	Signal anchor	–	
	TA03740	TP03_0283	Papain homolog (*Theileria*-type)	MER000673	Cysteine proteinase precursor		1	–	0	4	4	Signal anchor	–	
	TA15665	TP02_0883	Dipeptidyl-peptidase I (*Plasmodium*-type)	MER050482	Cathepsin-like cysteine protease, putative		0	–	3	0	0	Signal peptide	Apicoplast	
	TA04105	TP03_0357	Dipeptidyl-peptidase I (*Plasmodium*-type)	MER050503	Cysteine proteinase, putative		0	–	0	0	0	Signal peptide	Secretory	
	TA10955	TP04_0598	ECP-1 peptidase	MER050511	Papain-family cysteine protease, putative		0	Yes	0	2	0	Signal peptide	Secretory	
	TA11565[n]	TP02_0140	Subfamily C1A nonpeptidase homologs	MER050486	Cysteine proteinase, putative		1	–	12	24	28	Signal anchor	–	

	TA15715	TP02_0098	Subfamily C1A unassigned peptidases	MER050487	Cysteine protease, putative	1	–	0	0	0	Signal anchor	–
	TA15660	TP02_0097	Subfamily C1A unassigned peptidases	MER050488	Cysteine protease, putative	1	–	0	0	0	Signal anchor	–
Other families												
Pitrilysin family (**M16**)	TA08635	TP04_0473	Subfamily M16B unassigned peptidases	MER048292	M16 peptidase, putative	1	–	0	2	0	Signal anchor	Apicoplast
Pitrilysin family (**M16**)	TA12185	TP02_0260	Subfamily M16C unassigned peptidases	MER053056	Falcilysin, putative	0	–	4	9	2	Signal anchor	Mitochondrion
O-Sialoglycoprotein peptidase (*M. haemolytica*) (**M22**)	TA11230	TP04_0852	Family M22 unassigned peptidases	MER053068	Glycoprotease, putative	1	–	1	0	0	Signal peptide	Apicoplast
Methionyl aminopeptidase 1 (*E. coli*; **M24**)	TA09495	TP03_0462	Subfamily M24A unassigned peptidases	MER080180	Methionine aminopeptidase, putative	0	–	0	0	0	Signal peptide	Apicoplast
Ste24 endopeptidase family (**M48**)	TA15890	TP02_0928	Ste24 peptidase	MER050480	Metallo-protease, putative	6	–	6	0	0	–	Apicoplast
Ste24 endopeptidase family (**M48**)	TA15870[n]	TP02_0929	Family M48 nonpeptidase homologs	MER050481	Metallo-protease, putative	4	–	4	4	10	–	Secretory

(*Continued*)

Table 11.2 (*Continued*)

Family[a]	*T. annulata* (gene)[b]	*T. parva* (locus)[c]	Peptidase or homolog (subtype)[d]	MERNUM[e]	*T. annulata* annotation[f]	TMHMM[g]	GPI anchor[h]	Schizont EST hits[i]	Merozoite EST hits[j]	Piroplasm EST hits[k]	SignalP3.0[l]	Predicted location[m]
ClpP endopeptidase family (**S14**)	TA05070[n]	TP03_0530	Family S14 nonpeptidase homologs	MER050505	ATP-dependent Clp protease proteolytic subunit	0	–	*0*	4	0	Signal peptide	Apicoplast
ClpP endopeptidase family (**S14**)	TA09390	TP04_0168	Family S14 unassigned peptidases	MER050508	CLP protease, putative	0	–	5	0	0	–	Apicoplast
Signal peptidase I family (**S26**)	TA18300	TP03_0804	Signalase (eukaryote) 21 kDa component	MER048290	Signal peptidase, putative	2	–	13	0	0	Signal anchor	–
Signal peptidase I family (**S26**)	TA16115	TP01_0970	Subfamily S26A unassigned peptidases	MER050471	Mitochondrial membrane protease, subunit 2, putative	0	–	0	0	0	Signal anchor	–
Proteasome family (**T1**)	TA10875	TP04_0618	Proteasome subunit beta 3	MER050512	Proteasome beta 3 subunit, putative	0	–	0	0	0	–	Secretory
Prenyl protease 2 family (**U48**)	TA18155	TP03_0770	Family U48 unassigned peptidases	MER055353	Possible protease	7	–	0	0	0	Signal peptide	Secretory
Nonsecreted transmembrane proteins												
Methionyl amino-peptidase 1 (*E. coli*; **M24**)	TA21055[n]	TP01_0364	Family M24 nonpeptidase homologs	MER080179	Proliferation-associated protein 2g4, putative	1	–	3	2	3	–	–

FtsH endopeptidase family (**M41**)	TA16460	TP01_1122	Family M41 unassigned peptidases	MER053048	Metallo-protease/cell division cycle protein (FtsH homolog), putative	1	–	2	0	0	–	–
FtsH endopeptidase family (**M41**)	TA08725	TP04_0494	Family M41 unassigned peptidases	MER053064	Metallo-peptidase, putative	1	–	0	0	0	–	Mitochondrion
Ste24 endopeptidase family (**M48**)	TA15895[n]	TP02_0926	Family M48 nonpeptidase homologs	MER050479	Metallo-protease, putative	5	–	4	3	9	–	Mitochondrion

[a]Protease family as designated in the MEROPS database [135].
[b]*T. annulata* gene identifier, http://www.genedb.org/.
[c]*T. parva* locus identifier, http://www.tigr.org/tdb/e2k1/tpa1/.
[d]MEROPS annotation.
[e]MEROPS identifier.
[f]*T. annulata* annotation [133].
[g]Predicted number of transmembrane domains determined using an algorithm based on a hidden Markov model (Krogh *et al.*, 2001), http://www.cbs.dtu.dk/services/TMHMM/.
[h]Glycosyl-phosphatidylinositol (GPI) anchored proteins identified using DGPI v. 2.04, http://129.194.185.165/dgpi/DGPI_demo_en.html.
[i]Macroschizont ESTs mapping to gene (from a total of 5106 macroschizont-derived ESTs).
[j]Merozoite ESTs mapping to gene (from a total of 3101 merozoite-derived ESTs).
[k]Piroplasm ESTs mapping to gene (from a total of 4540 piroplasm-derived ESTs).
[l]SignalP3.0 was used to identify signal peptide and signal anchor sequences, http://www.cbs.dtu.dk/services/SignalP/.
[m]The target location of each protein was predicted using TargetP1.1, http://www.cbs.dtu.dk/services/TargetP/ and PATS, http://gecco.org.chemie.uni-frankfurt.de/pats/pats-index.php.
[n]*Nonpeptidase homologs.*

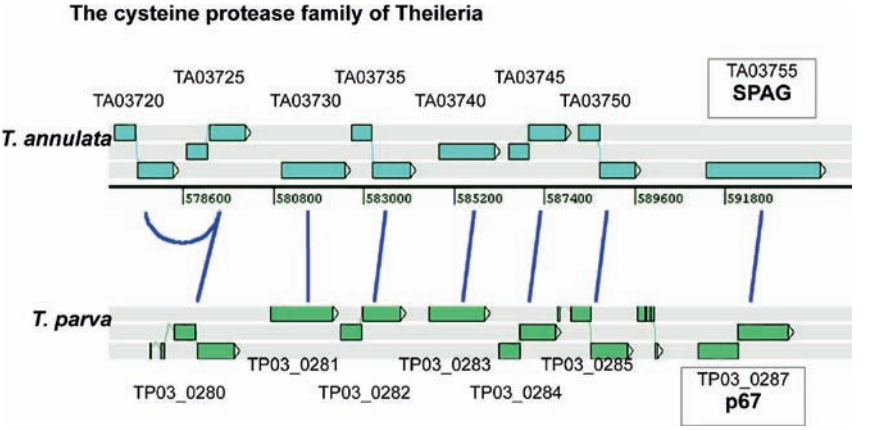

Figure 11.3 Synteny of chromosome 3 in *T. annulata* and *T. parva* at the cysteine protease locus, upstream of the sporozoite surface antigen-encoding gene SPAG/p67.

for *T. annulata*, 31% possess a signal peptide, a figure compatible with that computed for *P. falciparum* (35%), indicating that significant expansion of *Theileria* secreted peptidases in relation to immortalization of the host leukocyte has not occurred. This is supported by failure to unequivocally identify a *Theileria* peptidase that is predicted to be secreted into the host cell and expressed specifically by the transforming macroschizont stage (although three potential candidates lack expression data). Indeed, the majority of peptidases with a signal peptide are either predicted to be located in the apicoplast (suggesting a requirement for polypeptide processing in this organelle) or targeted to parasite membrane. Predicted membrane peptidases include an expanded family of cysteine protease genes (Figure 11.3) present in both *T. annulata* (seven tandemly organized genes) and *T. parva* (six tandemly organized genes) that are likely to have an important function. However, the expression pattern of these genes indicates that their function will not be specific to, or required by, the macroschizont-infected leukocyte. In contrast, one membrane peptidase (TA18300) shows evidence of high-level macroschizont-specific expression. This protein is highly likely to play a role in parasite-mediated control of host cell phenotype, as it is predicted to function as a signal peptidase (21 kDa eukaryotic signalase). Cleavage of signal peptides by these peptidases [137] to allow translocation of proteins across the macroschizont membrane may be a critical component of the pathway that allows transport of parasite (secretome) proteins into the host cell compartment [133, 136]. While it remains to be investigated whether specific inhibition of the *Theileria* signalase could be achieved, members of this family of peptidases are known to be essential for many bacteria and are regarded as drug targets [138].

In addition to peptidases with signal peptides, a further 47 peptidases are predicted (data not shown). Of these, two groups that show preferential expression in the macroschizont stage are of interest. The first group that shows strong bias for expression by the macroschizont contains putative components of the proteosome,

while the second group encodes predicted ubiquitin carboxyl-terminal hydrolases. It seems plausible that the rapidly proliferating macroschizont stage has a significant requirement to control protein turnover by targeting polypeptides for degradation.

From our bioinformatic analysis, peptidases that could provide rational targets for drugs that are detrimental to the macroschizont-infected cell have been identified. These predictions are based on the validity of the algorithms employed and it is possible that potential candidates have been missed. For example, TA15665 has identity with cathepsin cysteine proteases that may play a role in regulation of metastasis, apoptosis or proliferation of cancer cells [139] and is specifically expressed by the transforming macroschizont. This protein is, however, predicted to be located to the apicoplast. Clearly if research into *Theileria* peptidases is to progress, studies using specific inhibitors on cultures of *Theileria*-infected cell lines *vs* the uninfected counterparts need to be performed and, ideally, a gene knockout system developed for this parasite.

Leishmania

Protozoan parasites of the genus *Leishmania* cause vector-borne diseases in vertebrates and contribute significantly to human morbidity and mortality worldwide. Human diseases caused by *Leishmania* spp. include cutaneous, mucocutaneous and visceral leishmaniasis. This kinetoplastid parasite has a relatively simple dimorphic life cycle consisting of promastigote and amastigote stages. *Leishmania* promastigotes multiply as spindle-shaped flagellates in the midgut of the insect vector, a phlebotomine sandfly. The parasite is transmitted to a human host when the biting sandfly takes in a blood meal. Within humans the parasites infect phagocytic cells of the immune system and differentiate into the amastigote stage, which is intracellular, ovoid and aflagellated. Depending on the species of *Leishmania* and the host's immune status, the parasites establish a cutaneous infection or spread to the viscera. These visceral infections, if untreated, result in hepatosplenomegaly and death. *Leishmania* infects approximately 12 million people in 88 countries. There are an estimated 1.5–2.0 million new cases annually and 350 million people at risk of infection [140]. Due to the lack of safe and effective treatments for this disease, leishmaniasis is classified by the World Health Organization as Tropical Disease Research Category I, an emerging or uncontrolled disease (http://www.who.int/tdr/).

The lack of an effective vaccine for leishmaniasis makes chemotherapy the primary method for controlling the disease. While there are several drugs currently used to treat leishmaniasis, the use of these drugs is limited significantly by cost, toxicity, and widespread resistance. For decades pentavalent antimonials have been the mainstay of leishmaniasis chemotherapy; however, these drugs are being rendered obsolete in some endemic regions due to increased parasite resistance [141]. Other important drugs for treatment of leishmaniasis include paromomycin (an aminoglycoside antibiotic), miltefosine (developed as an anticancer drug), amphotericin B (an expensive antifungal), and pentamidine. The latter two drugs are associated with

considerable side-effects. The molecular target of pentamidine remains unknown, although resistance to this drug in parasites is linked to intracellular changes in arginine and polyamine concentrations [142]. Interestingly, pentamidine has been shown to inhibit a putative virulence factor in trypanosomes – the cytosolic serine protease, oligopeptidase B [143]. Because proteases represent an intriguing set of chemotherapeutic targets in *Leishmania*, research is currently underway to elucidated which of these proteases are virulence factors and how best to rationally design or identify inhibitors.

While the vast majority of peptidase research in *Leishmania* focuses on the metallo- and cysteine proteases, recent studies have illustrated the importance of serine proteases and identified aspartic protease activity in this parasite. A *Leishmania* cathepsin D-like aspartic protease (Clan AA) has just been identified and is postulated to be important in promastigote proliferation. The aspartic protease inhibitor, diazoacetyl-norleucinemethylester was found to inhibit *L. mexicana* proliferation with an LD_{50} of 22 μM at 72 hours [144]. Several serine proteases have been identified in *Leishmania*, including homologs to those that are putative virulence factors in the trypanosomes. Serine protease inhibitors benzamidine, N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and a sea anemone-derived Kunitz-type inhibitor were all found to be leishmanicidal and induced changes in the ultrastructure of the parasite's flagellar pocket [145]. *L. amazomensis* oligopeptidase B (Clan SC) has recently been homology modeled. The three-dimensional model that was generated will serve as a basis for evaluating this enzyme as a target for structure-based drug design [146]. Ongoing research is continually expanding our knowledge of *Leishmania* serine proteases and demonstrating them to be potential drug targets. Cysteine proteases are abundant in *Leishmania* and have become potential chemotherapeutic targets as well as candidates for vaccine development. The metacaspase (Clan CD, a distant relative of caspase not found in mammals) of *L. major* has been reported to be essential for nucleus and kinetoplast segregation in replicating pro- and amastigotes. Metacaspase null parasites are not viable making this enzyme a potential drug target [147]. Multiple cathepsin B- and cathepsin L-like cysteine proteases (Clan CA) are also present in *Leishmania*. Gene disruption of the cathepsin L-like proteases of *L. mexicana* (*lmcpb* genes) has shown that, while these enzymes are not required for *Leishmania* replication or differentiation *in vitro*, they are virulence factors. Macrophage infectivity of *lmcpb* null parasites is reduced by 80% [148]. The amastigote-specific cathepsin L-like protease of *L. chagasi* (Ldccys2) has also been found to play a role in macrophage infection and intra-macrophage survival. Ldccys2 null parasites are inviable and antisense RNA inhibition of Ldccys2 reduces parasite virulence *in vitro* [149]. Recent evidence has indicated that *L. mexicana* cysteine proteases prevent mice from developing a protective Th1 response during infection. Therefore, inhibiting *Leishmania* cysteine proteases may prevent the parasite from suppressing the host's protective immune response [150]. Molecular models of *L. major* cathepsin B- and L-like proteases have been generated and used for *in silico* screening. Potential protease inhibitors were identified, including six with IC_{50}s below 50 μM against *L. major* cpB. Three of these inhibitors, hydrazide compounds, prevented parasite proliferation at 5–50 μM *in vitro* [151, 152].

Possibly the most studied *Leishmania* protein is the major surface glycoprotein (GP63), a metalloprotease (Clan MA). This enzyme is the most abundant surface protein on *Leishmania* and is expressed on promastigotes and amastigotes of all species studied. The crystal structure for this enzyme was solved over a decade ago [153]. GP63 has several known functions including the binding and breakdown of complement component C3, resulting in parasite resistance to complement-mediated lysis [154]. GP63 deficient mutants of *L. major* exhibit sensitivity to complement-mediated lysis and a delay in lesion formation *in vivo* [155]. Additionally, GP63 has been shown to protect *Leishmania* from antimicrobial peptides such as mammalian α- and θ-defensins, magainins, and cathelicidins [156]. These peptides represent an important arm of the innate immune system. Effective nontoxic inhibitors of GP63 are not readily available; however, novel peptide inhibitors of GP63 based on myristoylated alanine-rich C kinase substrate (MARCKS) have been developed with an ID_{50} of approximately 1 μM [157]. GP63 is clearly a major factor in *Leishmania* virulence; thus, identification and design of GP63 inhibitors are a high priority for the control and treatment of leishmaniasis.

Giardia

Giardia lamblia (syn.: *G. intestinalis*, *G. duodenalis*) is an enteric flagellated protozoan parasite that infects many vertebrates, including man; and it is singularly the most commonly isolated intestinal parasite globally, with over 20% of the world's population infected [158]. In addition to its medical significance, *Giardia* is also of interest to evolutionary biologists as it represents the deepest branching clade of the eukaryota [159]. The simple lifecycle of *Giardia* involves asexually dividing intestinal trophozoites that are responsible for the clinical symptoms of giardiasis, namely: diarrhea, abdominal pains, nausea, vomiting, malaise, and fever. A second stage, an inactive environmentally resistant and infective cyst stage, allows for transmission of the parasite to new hosts. The processes of cyst formation (encystations) and cyst rupture (excystation) involve clan CA cysteine protease [160, 161], and to date 27 members of this family have been identified [162, 163]. The most highly expressed clan CA protease is *G. lamblia* cathepsin B-like protease 2 (*Gl*CP2). Chemically targeting the cysteine endoprotease(s) essential in excystation would thwart a new infection; however, to date, no target protease has been validated.

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12
In Search of Trypanocidal Drugs

*Leopold Flohé**

Abstract

Protozoan parasites of the genera *Trypanosoma* and *Leishmania* are the causative agents of hard-to-treat diseases, such as Chagas' disease, African sleeping sickness, and the various forms of leishmaniasis. Taken together they account for more than 500 000 fatalities/year and also represent a major economic problem by affecting livestock. Prevailing in less-developed countries, these diseases present a desperate therapeutic scenario that demands improvement. Recent strategies try to identify and characterize parasite-specific molecular targets for rational drug design. In this context, the trypanothione system deserves particular consideration. In trypanosomatids, trypanothione substitutes for the mammalian redox mediator glutathione in the detoxification of hydroperoxides, heavy metals, drugs, and hydroxyaldehydes. For hydroperoxide detoxification, trypanothione reduces tryparedoxins, which in turn reduce peroxiredoxin-type and GPx-type peroxidases. Tryparedoxins are also essential for nucleic acid synthesis and regulate cell division. Several, though not all, components of the system proved to be pivotal for the viability and virulence of trypanosomatids. Suppression of trypanothione synthetase (TryS) by dsRNA interference impairs viability in unstressed *T. brucei* and rapidly kills the parasites under mild oxidative stress. Further aspects supporting the use of TryS as a drug target are low abundance and uniqueness of sequence. TryS inhibition is therefore considered to be a particularly attractive strategy to fight infections with African trypanosomes and likely those caused by other *Trypanosoma* and *Leishmania* species. A series of N^5-substituted paullones inhibits TryS at nanomolar concentrations, decrease trypanothione in *T. brucei*, impair viability, and thus is considered to provide leads for the development of trypanocidal drugs.

Introduction

The therapy of infectious diseases caused by protozoan parasites of the trypanosomatid family is a neglected area of research and drug development. These parasites

comprise the causative agents of African sleeping sickness (*Trypanosoma brucei rhodesiense*, *T. brucei gambiense*), Chagas' disease in Latin America (*T. cruzi*), and the various forms of leishmaniasis such as the fatal visceral leishmaniasis kala-azar, (*Leishmania donovani*, *L. chagasi*), Espundia or mucocutaneous leishmaniasis (*L. mexicana* complex, subgenus *Viannia*), cutaneous leismaniasis (*L. major*, *L. tropica*, etc.) and diffuse cutaneous leishmaniasis which may be caused by many *Leishmania* species, depending on the immune status of the patient. Altogether they affect more than 30 million people and account for half a million fatalities per year. Trypanosomes (*T. brucei brucei*, *T. evansi*, *T. vivax*) also cause substantial economic problems in endemic areas by affecting livestock [1], and, like the related Bodonidae, impose major hazards for fishery and aquaculture [2].

As can be learned from recent reviews [3] and the regularly updated publications of the WHO (http://www.who.int/factsheets/), treatment of such diseases, if available at all, is largely unsatisfactory in terms of safety and efficacy. This unsatisfactory therapeutic scene contrasts sharply with the therapeutic needs in terms of people at risk, number of patients affected, and associated fatalities. This discrepancy is primarily due to the prevalence of these diseases in tropical and subtropical countries of partially poor socioeconomic standards. Associated difficulties in market penetration at reasonable profit margins have dampened the engagement of major pharmaceutical companies. In consequence, effective new drugs have not been developed and those available still comprise old-fashioned toxic arsenicals (Melarsoprol, Mel B) and antimony-containing compounds (Pentostam), have unfavorable kinetics (Suramin, Germanin), or are unspecific redox cyclers that damage both host and parasite, such as the nitrofuran Nifurtimox (Lampit) and the nitroimidazol benznidazole (Radanil/Rochagan/Ragonil). More recent additions to the therapeutic armamentarium are: (i) spin-offs from cancer therapy, such as the ornithine decarboxylase inhibitor α-difluoromethylornithine (eflornithine or DFMO, Ornidyl, which is also active against African trypanosomiasis) and miltefosine (Impavido/Miltex, which *inter alia* is used in visceral leishmaniasis), or (ii) broad-spectrum antibiotics like the amino glycoside paromomycin (Humatin) or the macrolide amphotericin B (Abelcet). The pipeline of drugs for neglected diseases such as leishmaniasis and trypanosomiasis is virtually empty. In fact, out of the 1393 new drugs marketed from 1975 to 1999 only 13, or a mere 1%, were for tropical diseases in general.

The advance of genome sequencing of the major pathogenic species of the trypanosomatids family (*T. brucei*, *T. cruzi*, *T. congolense*, *T. vivax*, *L. major*, *L. infantum*, *L. braziliensis*) promised easy and fast therapeutic progress. But as in other fields, functional analysis of the genomes is lagging behind, which hampers efficient exploitation of the huge and steadily growing data bases. The availability of complete genome sequences, however, substantially accelerated the analysis of metabolic pathways that had for long attracted interest as target areas for therapeutic intervention. One example of this kind is the trypanothione system that appears to be uniquely established in Kinetoplastida with limited homology in the parasites' mammalian hosts [4–9] and possibly some other protists [10]. We therefore compile here what has been achieved in terms of functional characterization of the trypanothione system, the genetic validation of its components as potential drug targets, target structures, and structure-based drug design.

The Trypanothione System of Kinetoplastida as an Attractive Drug Target Area

Biological Context

Trypanothione, $T(SH)_2$, was first described in 1985 by Fairlamb and Cerami [11], identified as bis(glutathionyl)spermidine [12] and considered as cosubstrate of a "trypanosomal glutathione reductase" [12]. However, it soon turned out that trypanosomes only contain two enzymes that utilize glutathione, glutathionylspermidine synthetase (GspS) and trypanothione synthetase (TryS), while all other types of enzymes that are specific for GSH in mammals depend on trypanothione in the parasites [4, 6, 7, 13]. A typical example of a parasite-specific pathway is the metabolism of hydroperoxides, which in mammals is predominantly achieved by the GSH system, but in trypanosomes was found to be catalyzed by a unique cascade of redox reactions: It starts with trypanothione reductase (TryR), the enzyme first misnamed glutathione reductase. It regenerates reduced trypanothione. The latter reduces a thioredoxin-related protein called tryparedoxin (TXN) which, in turn, reduces a peroxiredoxin-type peroxidase (TXNPx) [14]. This basic scheme of hydroperoxide metabolism has meanwhile been significantly amended (see below) and also provides a link to another important metabolic area. The parasite-specific protein TXN, which is the physiological target of $T(SH)_2$, proved to be the substitute of the pleiotropic redox mediator thioredoxin. In trypanosomes TXNs do not only mimic Trx in interacting with peroxiredoxins, they also reduce glutathione peroxidase-type proteins. Moreover, TXNs appear to substitute for thioredoxin in ribonucleotide reduction and metabolic regulation [15].

The trypanothione system, as depicted in Figure 12.1, appears to be common to the entire Trypanosomatidae family, although discrete differences have been discovered even between species of the same genus, for example, in trypanothione synthesis: While in *T. brucei* and some species of the genus *Leishmania* such as *L. braziliensis* and *L. major* TryS is the only enzyme involved, *C. fasciculata*, *T. cruzi*, and *L. infantum* have a GspS on top of a TryS. As a rule, TryS can catalyze both steps of trypanothione biosynthesis, while GspS only catalyzes the first step, that is, the formation of Gsp. Gsp may substitute for $T(SH)_2$ in some essential metabolic functions as, for example, in hydroperoxide metabolism and ribonucleotide reduction [16]. Moreover, TryR can accept Gsp disulfide as substrate to regenerate the thiol form. Further, the ascorbate peroxidase is not always present and the glyoxalase system appears to be abortive in some species.

Validation of Potential Drug Targets

Circumstantial Evidence for the Therapeutic Relevance of the Trypanothione System

Inhibition of the trypanothione system may be considered as a clinically validated therapeutic approach, since it is the mechanistic basis of approved trypanocidal drugs: DFMO inhibits an early step in the biosynthesis of spermidine and thus of

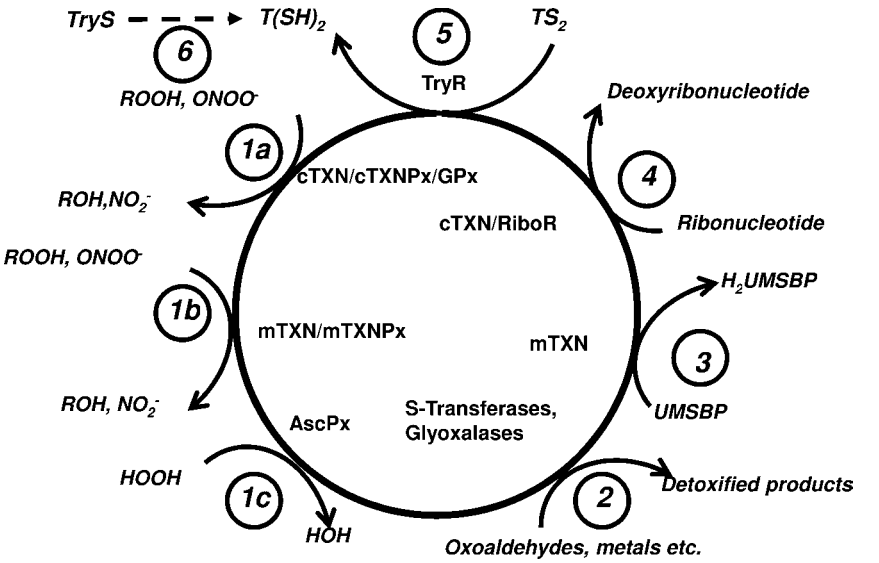


Figure 12.1 Functions of the trypanothione system.Trypanothione is essential for the entire hydroperoxide metabolism of trypanosomatids, as the physiological reductant of tryparedoxins (TXN; 1a) [17], which exist as cytosolic (cTXN) and mitochondrial forms (mTXN; 1b). TXNs provide the reduction equivalents of the peroxiredoxin-type tryparedoxin peroxidases (TXNPx), which exist as cytosolic (cTXNPx, 1a) and mitochondrial forms (mTXNPx; 1b) [18–20] and act on a broad spectrum of hydroperoxides, including peroxynitrite [14, 21–25]. TXN (and not glutathione) is also the physiological reductant of glutathione peroxidase-type peroxidases (GPx; 1a) [26]. $T(SH)_2$ is required to recycle ascorbate [27] (1c), the substrate of the only heme peroxidase in *T. cruzi* and *Leishmania major*. $T(SH)_2$ is involved in detoxification of heavy metals [28], oxo-aldehydes via the glyoxalases system [29–31], and xenobiotics by an S-transferase [32, 33] (2). $T(SH)_2$, again mediated by mTXN, reduces the universal minicircle sequence binding protein (UMSBP) (3), which is believed to be indispensable for kinetoplast replication [34]. $T(SH)_2$ directly, but more efficiently via TXN, reduces ribonucleotide reductase (4) and is therefore indispensable for DNA biosynthesis and repair [16]. In these reactions (except for the detoxification of heavy metals, oxo-aldehydes, and xenobiotics), $T(SH)_2$ is oxidized and has to be regenerated by a typical flavin-containing disulfide reductase (5), trypanothione reductase (TryR). *De novo* synthesis of $T(SH)_2$ (6) is achieved by stepwise ligation of two glutathionyl residues to spermidine with consumption of two moles of ATP [4]. Depending on species, this biosynthesis is catalyzed by two enzymes, glutathionylspermidine synthetase (GspS) and trypanothione synthetase (TryS), or by one enzyme catalyzing both steps (TryS) [4, 35–41].

trypanothione, the decarboxylation of ornithine to putrescine. Interestingly, its therapeutic use is largely confined to African sleeping sickness, because *T. cruzi* as an intracellular parasite can take up spermidine from the host cell and therefore is resistant to DFMO [42]. Trivalent arsenicals react with trypanothione itself, with trypanothione reductase or tryparedoxin, and in consequence are trypanocidal [43]. The mechanism of action of Chagas' drugs like nifurtimox or benznidazole is not precisely known, but they provoke an oxidative stress which has to be counteracted by the trypanothione-dependent peroxidase system [44]. The unsatisfactory safety of the

Table 12.1 Genetic manipulations of trypanothione-related enzymes to reveal essentiality.

Enzyme	Species	Methodology	Phenotype
TryR	*T. cruzi*	Overexpression	Unchanged susceptibiliy to H_2O_2 [46]
TryR	*T. brucei*	Conditioned knockout	Impaired growth and viability, increased sensitivity to H_2O_2, less virulent in mice [47]
TryR	*L. donovani*	Overexpression	Unchanged susceptibiliy to H_2O_2 [46]
TryR	*L. donovani*	Dominant negative	Decreased virulence, no survival in macrophages [48]
TryS	*T. brucei*	siRNA	Impaired growth and viability, increased sensitivity to H_2O_2 and t-butyl hydroperoxide [17, 49]
cTXN	*T. brucei*	siRNA	Impaired cell growth and viability, enhanced sensitivity to H_2O_2 [50, 51]
mTXN	*T. brucei*	siRNA	None [51]
Thioredoxin	*T. brucei*	si RNA	None [52]
cTXNPx	*T. cruzi*	Overexpression	Enhanced resistance to H_2O_2, t-butyl hydroperoxide [20] and peroxynitrite [53]
cTXNPx	*T. brucei*	siRNA	Impaired cell growth and viability, increased sensitivity to H_2O_2 and t-bOOH [51]
cTXNPx	*L. amazonensis*	Antisense RNA; overexpression	Altered hydroperoxide and arsenite resistance [54]
mTXNPx	*T. cruzi*	Overexpression	Enhanced resistance to H_2O_2, t-bOOH[20] and peroxynitrite [53]
mTXNPx	*T. brucei*	siRNA	None [51]
mTXNPx	*L. infantum*	Knockout	None [55]
GPx-I	*T. cruzi*	Overexpression	Enhanced resistance to H_2O_2 and t-butyl hydroperoxide [56]
GPx-l	*T. brucei*	siRNA	Impaired growth and viability, increased susceptibility to H_2O_2 [26, 51]
APx	*T. cruzi*	Overexpression	Enhanced resistance against H_2O_2 [27]
Glyoxalase II	*T. brucei*	siRNA	None [29]

available drugs results from a lack of specificity (arsenicals, antimony drugs), redox cycling (nitrofurans, nitroimidazols), or inhibition of the system at sites that are shared with the mammalian metabolism (for instance ornithine decarboxylation by DFMO) [45]. Accordingly, more recent drug discovery efforts focused on specific inhibition at pivotal sites of the system, such as TryR or TryS.

Genetic Drug Target Validation

The importance of the trypanothione system for parasite survival has been addressed with different genetic technologies and at different enzymatic levels [7]. The outcome of related knockout and knockdown experiments is compiled and referenced in Table 12.1.

Impaired hydroperoxide metabolism was consistently observed when TryR, cytosolic forms of TXN and TXNPx, and, as demonstrated in Figure 12.2, TryS were

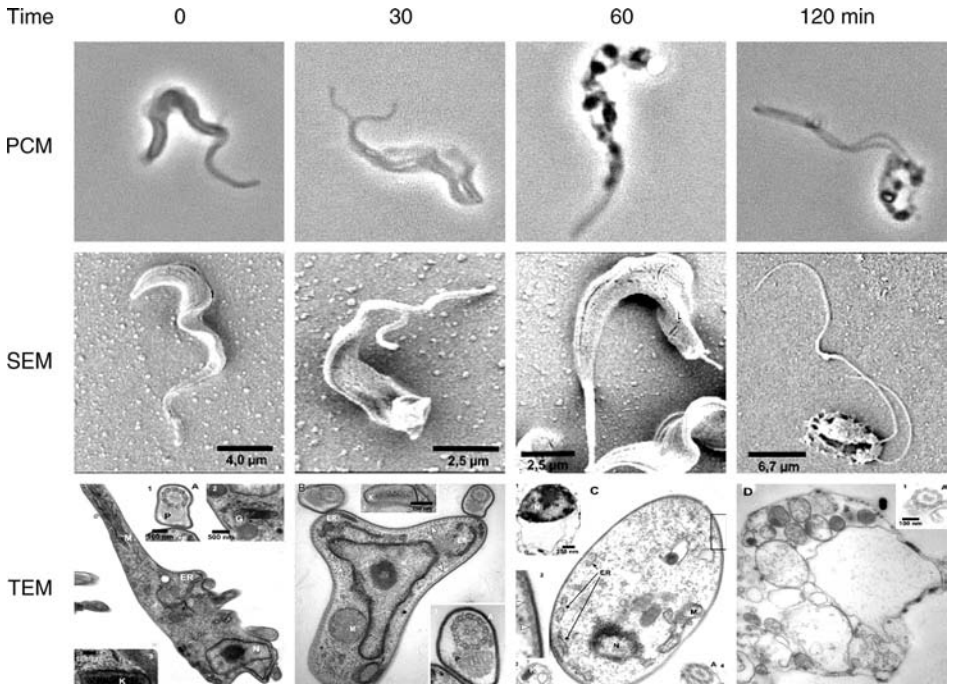


Figure 12.2 Sensitivity of TryS-deficient *T. brucei* to H_2O_2.TryS was knocked down by dsRNAi 24 h before exposure to an H_2O_2 flux of 60 pM/ml generated by glucose plus glucose oxidase. At time zero (0 min), when the T(SH)2 content had dropped to ~10% of controls, the parasites are still growing and look normal (PCM, phase contrast microscopy; SEM, scanning electron microscopy; TEM, transmission electron microscopy). After 30 min of H_2O_2 exposure, detachment of flagella and swelling of intracellular organelles are seen, proceeding with the appearance of blebs and wrinkles (60 min) and ending in complete disintegration after 120 min. Pictures were produced by H. Lünsdorf; data are from Ref. [17].

decreased. Surprisingly, however, the downregulation of the mitochondrial TXN and TXNPx in *T. brucei* did not yield any obvious phenotype and also the knockout of the latter enzyme in *L. infantum* did not display any increased sensitivity to hydroperoxide when added to the medium. The physiological relevance of increased hydroperoxide sensitivity became evident from conditional knockout of TryR in *T. brucei* and a dominant negative approach in *L. donovani*: The parasites lost virulence [47] and could no longer survive in macrophages [48]. Also, the $T(SH)_2$ content in *T. cruzi* appears to correlate with clinical virulence and resistance against pro-oxidant drugs [53]. The findings underscore the pivotal role of the trypanothione system for the detoxification of H_2O_2, organic hydroperoxides, and peroxynitrite; and they further corroborate the relevance of the parasites' hydroperoxide metabolism to virulence.

A second phenomenon, in particular observed when cTXN, TryR or TryS was knocked out or knocked down, is impairment of growth, up to complete growth

arrest. However, loss of viability in unstressed parasites and spontaneous dying is not consistently observed just by knocking down cTXN [50]. Similarly, the conditional knockout of TryR only impaired the viability of the parasites when the enzyme activity was reduced to less than 5% of controls [47]. In contrast, knockdown of TryS killed *T. brucei* even at a residual $T(SH)_2$ content of 10–15% of controls, but only if these conditions were kept for 48 h [17]. The observations reveal that the unstressed parasites are able to survive for considerable time without an intact antioxidant defense, but finally die from disturbances distinct from oxidative stress, likely from shortage of deoxynucleotides. Interestingly, knocking down thioredoxin, which is present in *T. brucei* [57] and in most species is the physiological substrate of ribonucleotide reductase, did not yield any phenotype, suggesting that in trypanosomatids tryparedoxin has adopted the pivotal role of thioredoxin in nucleic acid metabolism [7].

Convincing proof of essentiality is still lacking for trypanothione S-transferase, the glyoxalase system, UMSCP, ribonucleotide reductase GspS. It is also evident from Table 12.1 that most of the target validation work has been performed by means of double-strand RNA interference in *T. brucei*, a technology that has so far proven to be inapplicable for other *Trypanosoma* and *Leishmania* species [58]. Possible species differences in essentiality of the various system components have therefore to be considered. For instance, ribonucleotide reduction must theoretically be rated as generally required. This does, however, not necessarily imply that the preferred way of ribonucleotide reduction via the trypanothione/TXN couple, as demonstrated in *T. brucei* [16], is mandatory for all trypanosomatids, since GSH, Gsp or low levels of Trx may suffice to guarantee a satisfactory reduction of RiboR, and in consequence, the supply with DNA building blocks. Further, an active salvage pathway might bypass ribonucleotide reduction in intracellular parasites. A compensation of $T(SH)_2$ by Gsp may also be envisaged in species equipped with both GspS and TryS. These and other possible complications reveal that the advanced target validation work in *T. brucei* can unfortunately not be generalized; and they underscore the necessity to investigate the system in other trypanosomatid species by more tedious techniques to identify the enzymes that are equally important in the entire family of parasites.

Druggability of Individual Targets

The term "druggability" addresses the question to what extent a particular target can be selectively inhibited under consideration of structural and mechanistic features [59].

An almost typical case of a nondruggable target is TXN:

- TXNs are globular proteins without any obvious substrate binding pocket.
- Site-directed mutagenesis [60], NMR binding studies with substrate homologs [61], and molecular modeling [62, 63] reveal that their interaction with substrates are primarily achieved by surface-exposed charged residues.

- Mechanistically, TXNs operate by an enzyme substitution mechanism, which implies that essentially the same active site has to interact with both substrates, the flexible peptide trypanothione and large proteins such as TXNPx.
- In biological terms, TXNs, like Trx, are pleiotropic; that means they have to reduce a variety of in part unrelated protein substrates.

Taken together, these characteristics almost exclude the possibility to inhibit TXN selectively. In line with this conclusion, the close $T(SH)_2$ homolog bis (ophthalmyl)spermidine competitively inhibited TXN with an inhibition constant of just 1 mM.

Druggability of the peroxiredoxin- or GPx-type peroxidases does not appear much better. The reduced enzymes expose the sulfurs of their reactive cysteine residues (C_P) at the bottom of a flat well. This active site architecture allows access of a large variety of hydroperoxides. The reaction centre of the oxidized enzymes is a disulfide bridge between C_P and the "resolving cysteine" (C_R) with the sulfur of C_R exposed to a poorly structured surface [64, 65], again not an ideal prerequisite for the design of selective inhibitors.

In contrast, the TryR structures display a characteristic substrate binding pocket which markedly differs from that of mammalian homologs such as glutathione reductase or thioredoxin reductases [28, 66–69]. Selective inhibition therefore is possible and has indeed been achieved by means of different pharmacophores [69–74] (see below).

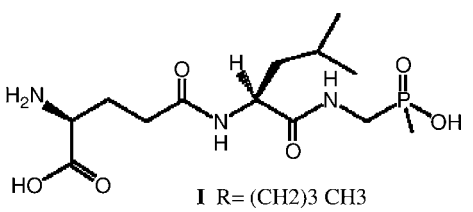
Unfortunately, the TryS structure has just recently been resolved [75]. Therefore, in the past inhibitor design was largely guided by analogy to substrates. A molecular model of the TryS structure based on the homologous GspS of *E. coli* [76] has recently been constructed (M. Comini, unpublished data). It essentially confirms the assumption that TryS (and also GspS) may be considered to belong to the ATP-grasp proteins [36]. This type of ATP-dependent ligases tends to squeeze the substrate ATP between two protein loops in a way that it can not be released before its γ-phosphate is cleaved. In consequence, ATP homologs that are bound similarly are not competed out by the abundant cellular ATP. This phenomenon has been successfully exploited for the design of inhibitors for protein kinases which behave similarly [77]. Embarking on this analogy, a novel class of TryS inhibitors was discovered, N^5-substituted "paullones," 2-(6-oxo-6,7-dihydro-5 *H*-benzo[2,3]azepino [4,5-b] indol-5-yl) -acetamides [78]. Paullones represent a class of ATP analogs that are known so far to inhibit protein kinases [79, 80], in particular cyclin-dependent kinases and glycogen synthase kinase [81, 82], but also display antileishmanial activity [83]. The TryS inhibitors based on the paullone scaffold inhibited the TryS of *C. fasciculata* with an IC_{50} as low as 30 nM and, as expected, the inhibition was not reversed by physiological concentrations of ATP or any other substrate. Inhibition of TryS and GspS has also been obtained by homologs of the substrate GSH and phosphinic acid derivatives thereof presumed to mimic the tetrahedral transition state that is formed during catalysis [84]. Taken together, progress suggests that the structures of TryS and GspS should be characteristic enough to achieve specific inhibition.

Experience with Trypanothione-Related Inhibitors in Biological Systems

So far only inhibitors of TryR and TryS have been investigated for trypanocidal activity. As a rule, the results were less promising than anticipated and none of them has been rated as promising enough to merit a preclinical or even clinical drug development. The compounds available so far can therefore at best serve as leads for optimization projects. Some revealing observations are, however, worth mentioning.

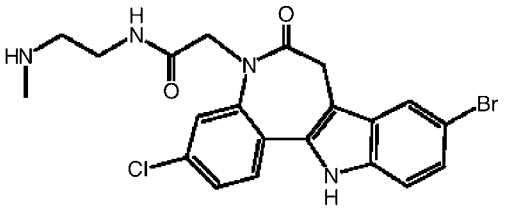
Reversible and "clean" inhibitors of TryR have so far proven to be disappointing *in vivo* irrespective of their activity at the enzymatic level, which is not surprising considering the necessity to inhibit the enzyme persistently by more than 95% to achieve any significant disturbance of the parasite's redox balance. By means of irreversible inhibition of TryR pronounced trypanocidal activities can be achieved. For instance, (2,2′ : 6′,2″-terpyridine)platinum(II) complexes, which inactivated *Tc*TryR at considerable rate constants up to $1.3 \times 10^4\,M^{-1}\,s^{-1}$, likely by specifically modifying the active site Cys52 (in the *T. cruzi* enzyme), also killed *Trypanosoma* and *Leishmania* species *in vivo* [70]. Also, unsaturated Mannich bases were shown to irreversibly inactivate trypanothione reductase from *T. cruzi* by alkylating Cys52 and showed strong trypanocidal activity [72]. Palladium complexes such as Pd(5-nitrofuryl-3-acroleine thiosemicarbazone)2 proved to irreversibly inhibit TryR of *T. cruzi* and to simultaneously impose an oxidative stress by redox cycling and accordingly were active *in vivo* [74]. Similarly, 1,4-naphtoquinones serve as "subversive substrates" of TryR, whereby redox cycling with continuous formation of superoxide radical is initiated which leads to inhibition of TryR and liponamide dehydrogenase associated with trypanocidal activity [85].

According to analogous inverse genetics, TryS inhibitors should have a more realistic chance to display *in vivo* activity. Amongst the GSH derivatives, a series of N-S-blocked glutathione monoester and diester derivatives based on N-benzyloxycarbonyl-S-(2,4-dinitrophenyl)glutathione displayed activity against *T. brucei brucei*, *T. cruzi*, and *L. donovani*, with an LD_{50} down to 0.4 μM [86]. Also, the series of phosphinopeptides that was synthesized to mimic the activated GSH [84] yielded lead compounds active enough to consider further development. Compounds I and II, in which L-γ-Glu-L-Leu-Gly replaces the glutathionyl residue, were found to inhibit *T. cruzi* amastigotes growing in myoblasts. The peptidic part of all these molecules, however, is likely amenable to fast degradation by peptidases.



I R= (CH2)3 CH3

II R= (CH3)4CH3



III

Of the paullone-based TryS inhibitors [78], the so far most active one, Compound III, was tested for trypanocidal activity in *T. brucei* and *T. cruzi*. For *T. brucei* an LD_{50} of 2 µM, measured after a 150 min exposure, was extrapolated; while all parasites had died after a 20 h exposure over the whole range of concentrations applied (10–100 µM; M. Comini, unpublished data). Surprisingly, however, the compound did at best marginally affect the growth rate of *T. cruzi* up to 100 µM (F. Irigoin and R. Radi, unpublished data). The reason for this discrepancy remains to be worked out. It may be due to differences in the TryS sensitivity between the two species or, more likely, to metabolic adaptation of *T. cruzi*. As mentioned above, *T. cruzi* is equipped with GspS which may compensate for a $T(SH)_2$ shortage by overproduction of Gsp to sustain its redox balance. If this case turns out to be true, the ideal drug should inhibit both TryS and GspS, which is not an unrealistic concept in view of the similarity of these enzymes in terms of sequence, structure, and substrate specificity.

Conclusion

The original concept that led us to think of inhibitors of the trypanothione system was to inhibit the defense system that protects pathogens against the oxidative challenge exerted by phagocytes during the innate immune response. Thereby the pathogens would be left unprotected against the killing reaction, which is mediated by O_2^{-}, H_2O_2, and peroxynitrite, and would lose viability and virulence due to oxidative damage [87]. The trypanosomes provided an excellent chance to evaluate the validity of this concept, since their antioxidant defense system differs markedly enough from that of the mammalian hosts to enable a selective inhibition. When it became obvious that trypanothione is required also for other vital functions in the parasites, the idea seemed even more attractive.

With growing knowledge, however, the realistic options for an efficient therapeutic intervention along these lines are shrinking. The TXNs, peroxiredoxins, and GPx-type peroxidases have close and in part essential relatives in mammals, and the chances to hit the parasitic congeners selectively must be rated as very low in view of the structural features and poor substrate specificities common to these classes of enzymes. Two options remain: blocking $T(SH)_2$ regeneration via TryR, and inhibition of the biosynthesis of $T(SH)_2$. Both strategies have been

validated according to the state of the art by inverse genetics, although not in all clinically relevant pathogens. Both strategies have been supported by inhibitor studies. The associated difficulties are equally obvious: (i) to expect a therapeutic result, TryR has to be inhibited almost completely; (ii) inhibition of TryS, depending on the species, may not suffice to critically affect the parasites. There is further ample evidence that these two approaches could be substantially fortified by imposing an oxidative stress; and it may be questioned whether this is sufficiently contributed by the innate immune response itself under the variable disease conditions.

Faced with these problems, one may guess that heading for the "clean" drug that just targets one particular enzyme may not hold the greatest promise. Synergism may certainly be anticipated from combining the two or more therapeutic approaches and, as in other hard-to-treat diseases, such multidrug treatment might be necessary to prevent the fast development of resistance. Finally, one should probably not be over-critical with respect to a clean mechanism of drug action. A bit of potentially hazardous redox cycling, as associated with some TryR inhibitors or subversive substrates, might not only be acceptable in fighting life-threatening diseases, but could even become the pivotal amendment to the innate immune response for parasite clearance.

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13
Trypanosomatid Protein Kinases As Potential Drug Targets

*Martin Wiese, Andrew Morris, and Karen M. Grant**

Abstract

The trypanosomatid protozoa include the medically important parasites *Trypanosoma brucei*, *T. cruzi*, and *Leishmania*. They are responsible for a wide range of diseases which blight the developing nations of the world. Current chemotherapy to treat these diseases is far from ideal and many different approaches are being taken to identify new drug targets. One family of potential drug targets are the protozoan protein kinases. Protein kinases are ubiquitous in eukaryotes and act in many different intracellular signaling pathways affecting for example: differentiation, proliferation, motility, and apoptosis. Within parasitic protozoa, several protein kinases play essential roles and disruption of their activity is seriously deleterious to the parasite. Moreover, despite homology to their mammalian counterparts, protozoan protein kinases are significantly different in ways which open up the possibility of developing parasite-selective inhibitors. In this chapter, we outline the current knowledge of important parasite protein kinases and discuss their suitability as novel drug targets.

Introduction

Diseases caused by trypanosomatid parasites have a significant impact on the world's population. The World Health Organization (WHO) estimates that there were 50 000–70 000 cases of African sleeping sickness caused by *Trypanosoma brucei* in 2005 (http://www.who.int/mediacentre/factsheets/fs259/en/). An estimated 12 million people are presently infected with *Leishmania* with two million new cases of leishmaniasis each year (http://www.who.int/leishmaniasis/burden/magnitude/burden_magnitude/en/index.html). Moreover, Chagas' disease, caused by *T. cruzi*, affected an estimated nine million people in 2006, predominantly children (http://www.who.int/mediacentre/news/notes/2007/np16/en/index.html). There

are no vaccines to prevent the spread of these diseases and the limited repertoire of drugs suffer from many drawbacks including toxicity, expense, requirement for hospitalization, and the emergence of drug-resistant parasites.

All three trypanosomatid protozoans, *Leishmania*, *T. brucei*, and *T. cruzi* have complex life cycles in which they progress through distinct morphological stages in both the invertebrate vector and mammalian host. In the mammalian host, *T. brucei* exists extracellularly in the bloodstream and cerebrospinal fluid, whereas *T. cruzi* and *Leishmania* are found intracellularly. *T. cruzi* can infect virtually every nucleated cell, while *Leishmania* infects reticulo-endothelial cells, such as macrophages and dendritic cells, and fibroblasts in late stages of the infection [1].

To survive, the parasite must adapt to the different environments within their invertebrate and mammalian hosts. The distinct phosphorylation patterns observed in the different developmental stages of these parasites suggest that both adaptation and proliferation are likely to be regulated by phosphorylation and dephosphorylation [2–8]. These distinct phosphorylation patterns must be dictated by differential protein kinase and protein phosphatase activity. Information on the signal transduction network of these parasites is still relatively scarce. Analysis of parasite genomes has identified 199 protein kinases in *L. major*, 190 in *T. cruzi*, and 176 in *T. brucei* [9], whilst the number of phosphatase catalytic domains was found to be 88 in *L. major*, 86 in *T. cruzi*, and 78 in *T. brucei* [10]. How these kinases and phosphatases are organized in signaling cascades is unknown, with only one example of interacting protein kinases published so far [11]. Comparative analyses of the kinome and phosphatome of the three trypanosomatid species have revealed certain differences between the parasites that might be related to their different life styles as intra- and extracellular pathogens. There are 20 kinases specific for *L. major*, 11 for *T. cruzi*, and three for *T. brucei* [9]. Some phosphatases present in *Leishmania* and *T. cruzi*, like protein phosphatase 6, are absent in *T. brucei*, possibly reflecting the extracellular life style of this parasite [10]. Moreover, some protein phosphatases of significant importance in higher eukaryotes, like the vertebrate mitogen-activated protein kinase phosphatases (MKP), are absent. Instead plant-like MKPs have been identified in all three trypanosomatids [10].

Potential drug targets against diseases caused by trypanosomatid parasites should be indispensable for parasite survival in the mammalian host. The relevant enzyme or protein could be involved in the proliferation, differentiation, and/or adaptation of the parasite to its environment in the mammal, for example, escape from the host immune response or change of metabolism according to the available nutrients. Protein kinases are thought to be involved in all three processes: proliferation, differentiation, and adaptation. Hence they are putative drug targets to treat trypanosomatid parasitic diseases.

Both eukaryotic protein kinases and atypical kinases have been found in all three trypanosomatid genomes [9]. Eukaryotic protein kinases (ePK) are characterized by the presence of 11 kinase subdomains containing a number of conserved amino acid residues. These are largely absent in the atypical kinases; however, some of the conserved amino acids can be found. The active site of ePKs is buried between two lobes: the amino-terminal lobe characterized by the presence of antiparallel β-sheets,

and a largely α-helical carboxy-terminal lobe [12]. Accessibility of the active site is often regulated by phosphorylation-induced conformational changes of the phosphorylation lip or activation loop [13]. Some ePKs require accessory molecules, like cyclins for the cyclin-dependent kinases, for full catalytic activity. Trypanosomatids possess ePKs that belong to the AGC group (PKA, PKG, PKC kinases), the CAMK group (calcium calmodulin-dependent kinases), the CK1 group (casein kinases), the CMGC group (cyclin-dependent kinases, mitogen-activated protein kinases, glycogen synthase kinases, cyclin-dependent kinase-related kinases), the STE group (homologs to "sterile" kinases from yeast), plus a group of nonclassified kinases [9]. Most trypanosomatid ePKs are serine/threonine kinases and some display features of dual-specificity kinases that can phosphorylate serine, threonine, and tyrosine residues. Interestingly, no tyrosine kinases or tyrosine kinase-like kinases were identified [9].

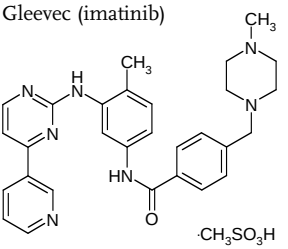
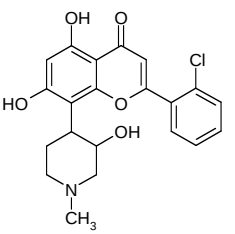
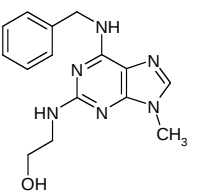
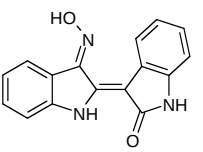
Protein kinases play crucial roles in the homeostasis of eukaryotic cells; and changes in their activity have been implicated in many human diseases, such as cancer, diabetes, neurodegenerative, and inflammatory disorders. Consequently, significant research has been undertaken to identify protein kinase-specific inhibitors that could be used to treat these diseases. Most small-molecule inhibitors of protein kinases interact with the ATP-binding pocket. The main drawback of this approach is that the ATP-binding site is relatively conserved, making it difficult to generate specific inhibitors that do not affect other kinases or even other ATP-binding proteins [14]. Moreover, only those inhibitors with a high binding affinity can compete with high intracellular ATP concentrations (10 mM). Newer types of inhibitors which either stabilize the inactive kinase conformation or inhibit substrate binding promise selective regulation of a specific protein kinase without interfering with the normal physiology of the cell [15]. The most prominent of this type of inhibitors is Gleevec (Table 13.1) which binds to the inactive form of Bcr-Abl kinase, platelet-derived growth factor receptor (PDGF-R), and stem cell factor receptor (c-Kit) and stabilizes the enzymatically inactive conformation without directly competing for ATP binding [16, 17].

The recent identification of protein kinases in the parasitic trypanosomatids and the elucidation that many are crucial for parasite survival or infectivity within the mammalian host opens up the possibility of discovering or developing pharmacological inhibitors of parasite protein kinases which may be useful as novel leads for antiparasite chemotherapy. In this chapter we outline what is already known about the role of certain key kinases and their suitability as future drug targets.

Mitogen-Activated Protein Kinases

Mitogen-activated protein (MAP) kinases and their signal transduction pathways play important roles in proliferation, differentiation, and adaptation in all eukaryotic cells. MAP kinase signal transduction cascades comprise a MAP kinase kinase kinase

Table 13.1 Pharmacological inhibitors of protein kinases.

Protein kinase inhibitor	Target kinase(s)	Mode of action
Gleevec (imatinib) 	abl (Abelson proto-oncogene) c-kit, cytokine receptor on haematopoietic stem cells PDGF-R (platelet-derived growth factor receptor)	Tyrosine kinase inhibitor, non-competitive with respect to ATP
Flavopiridol 	CDKs: CDK1, CDK2, CDK4 Protein kinase C	Serine/threonine kinase inhibitor, ATP-competitive
Olomoucine 	CDKs: CDK1/cyclin B CDK2/cyclin A CDK2/cyclin E CDK5/p35 MAPKs: ERK1	Serine/threonine kinase inhibitor, ATP-competitive
Indirubin 3′-monoxime 	CDKs: CDK1/cyclin B CDK2/cyclin A CDK2/cyclin E CDK4/cyclin D1 CDK5/p35 GSK-3β	Serine/threonine kinase inhibitor, ATP-competitive

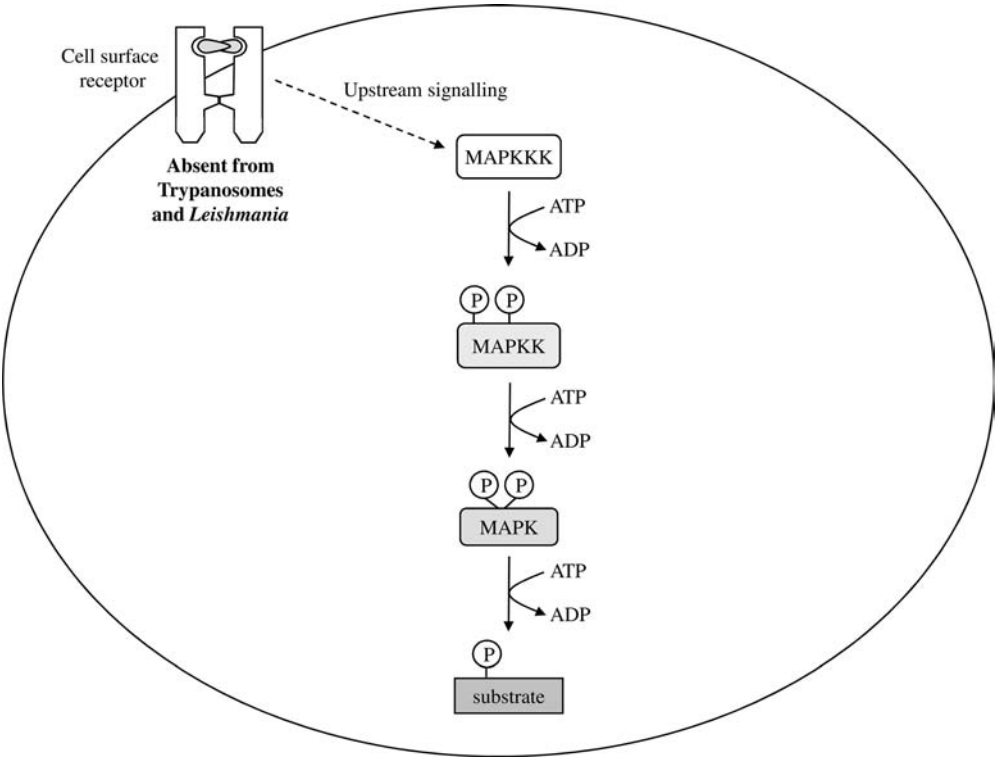


Figure 13.1 Mitogen-activated protein kinase signal transduction cascade of higher eukaryotes.MAP kinase signal transduction cascades comprise a MAP kinase kinase kinase (MAPKKK), activated by upstream signaling from a cell surface receptor, which phosphorylates a MAP kinase kinase (MAPKK) which in turn phosphorylates and activates a MAP kinase (MAPK). Homologs of most components of this pathway have been identified in Trypanosomes and *Leishmania*, with the notable exception of cell surface tyrosine kinases, which are absent from their kinomes [9].

(MAPKKK), which is activated by upstream phosphorylation relaying a signal from a cell surface receptor and phosphorylates a MAP kinase kinase (MAPKK) which in turn activates a MAP kinase (MAPK) by phosphorylation on the threonine and tyrosine residues in the TXY motif of the phosphorylation lip (Figure 13.1). In *Leishmania*, 15 putative MAP kinases, MPK1–MPK15, have been identified on the basis of sequence homology to MAP kinases from higher eukaryotes (Figure 13.2). With the exception of MPK7 and MPK8, clear homologs of the *Leishmania* kinases are also present in *T. brucei* and *T. cruzi*. Moreover, MPK7 and MPK8 are exceptional in carrying significant amino acid insertions in their phosphorylation lips, which extend the region between the HRD motif of the catalytic loop (containing the aspartate that functions as the catalytic base by accepting a proton from the attacking hydroxyl group of the substrate) and the TXY motif. The insertions extend this region to 80 and 100 amino acid residues, as compared to 30–38 amino acids in the other MAP kinases [8]. MPK7 has increased activity in *L. major* during differentiation from

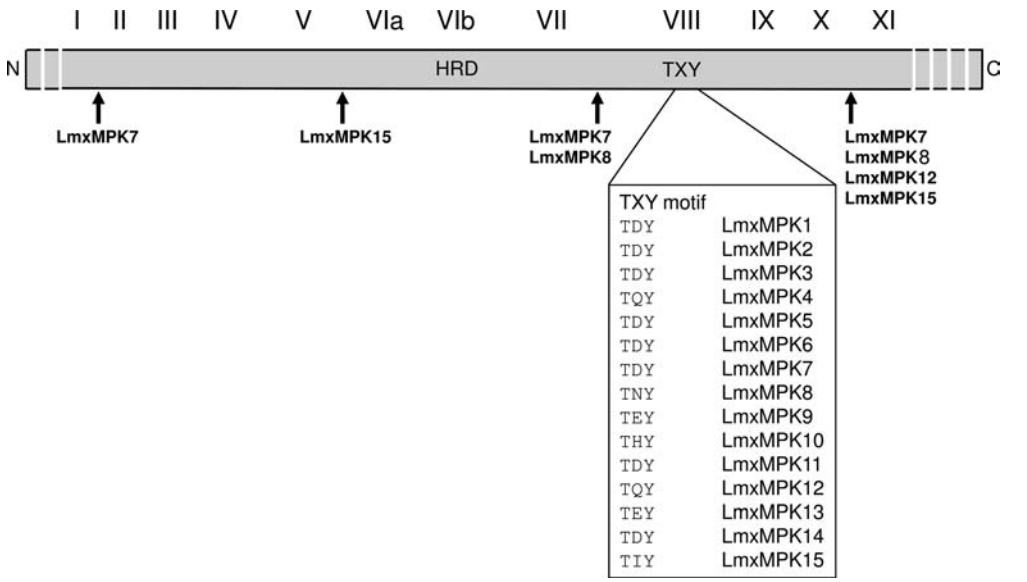


Figure 13.2 Structure of MAP kinases from *L. mexicana*.The bar represents the primary structure of a MAP kinase with extensions of variable length at both the N- and C-terminus. The Roman numerals indicate the conserved kinase subdomains. Positions of the HRD- and TXY-motifs are highlighted. Black arrows show sites of additional amino acid insertions in the indicated kinases. The different TXY phosphorylation motifs for LmxMPK1–LmxMPK15 are depicted.

promastigotes to amastigotes and in *L. donovani* amastigotes [8], implying that it might be important for the differentiation to, and maintenance of, the mammalian-infective amastigote stage. However, *L. mexicana* LmxMPK7 null mutants are still infective to Balb/c mice, albeit with a delayed lesion development (Bleicher and Wiese, unpublished data), which refutes the suitability of MPK7 as a drug target.

The kinase activity of MPK10 and MPK4 also appears to increase during the promastigote to amastigote differentiation step, implying a role for these two kinases in the process [8], but further experiments are required to corroborate the potential of MPK10 as a drug target. LmxMPK4 is essential for both the insect and mammalian life-cycle stages of *L. mexicana* [18]. No homozygous genomic null mutants could be obtained in promastigotes unless a wild-type copy of *LmxMPK4* was provided on a plasmid and this plasmid was retained in the absence of antibiotic selection. The recombinant parasites were used to infect Balb/c mice and amastigotes harvested from lesions retained the plasmid after more than a year without antibiotic selection. Clearly, LmxMPK4 is required for the proliferation of promastigotes and amastigotes and thus represents a suitable drug target. The *T. brucei* homolog, TbMAPK2, is involved in differentiation from bloodstream form trypanosomes to procyclic cells [19]. A null mutant displayed a delay in differentiation with subsequent cell cycle arrest but, unlike its *Leishmania* counterpart, TbMAPK2 is not required in the mammalian stage of the parasite and therefore is not a suitable drug target.

KFR1 (KSS1-, FUS3-related kinase 1) was the first MAP kinase homolog to be identified in trypanosomatids [20, 21]. Attempts to generate a null mutant for KFR1 in procyclic trypanosomes failed, indicating that the protein is essential in the insect stage of the parasite [20]. As its expression and activity is higher in trypanosomes in the bloodstream, it can be assumed that it is also essential in the mammalian-infective stage and therefore represents a potential drug target to treat African sleeping sickness, although further investigations using RNA interference are needed to validate its potential. A null mutant of LmxMPK1, the *L. mexicana* homolog of KFR1 (55.6% amino acid identity), is unable to proliferate in peritoneal macrophages and does not cause lesions in infected Balb/c mice [22], indicating the suitability of LmxMPK1 as an antileishmanial drug target. Moreover, it is highly conserved between different *Leishmania* species (92–97% amino acid identities between *L. braziliensis*, *L. infantum*, *L. mexicana*), implying that an inhibitor of MPK1 from all *Leishmania* species could be developed which would be effective against cutaneous, mucocutaneous, as well as visceral forms of the disease [23]. In fact, a similar level of cross-species conservation can be found for all *Leishmania* MAP kinases.

LmxMPK2 displays closest sequence similarity to LmxMPK1 (45% amino acid sequence identity). A null mutant for LmxMPK2 was also unable to cause lesions in infected Balb/c mice (Wiese, unpublished data), hence indicating that it is a suitable drug target. LmxMPK3 and LmxMPK9 are both involved in flagellar length regulation, as null mutant *L. mexicana* promastigotes display shorter or longer flagella than the wild type, respectively [11, 24]. However, neither mutant has impaired infectivity towards Balb/c mice, excluding them as anti-leishmanial drug targets.

Another interesting MAP kinase from *T. brucei* is TbMPK5. The TbMPK5 null mutant differentiated prematurely to the stumpy form in infected mice, leading to a reduction of the peak parasitemia [25]. Therefore, inhibition of this kinase could potentially alleviate the effects of the intermittent fever attacks in African sleeping sickness by reducing parasite numbers in the body. A null mutant for the *L. mexicana* homolog, LmxMPK5, persisted at the site of inoculation but did not cause lesions in infected Balb/c mice (Wanders and Wiese, unpublished data), implying that this kinase may be involved in amastigote proliferation and disease development. Inhibition of LmxMPK5 might limit the progression of leishmaniasis and promote the development of immunity against infection.

TbECK1 is a *T. brucei* MAP kinase homolog whose long C-terminal tail is important for regulation of the kinase's activity in the cell [26]. Inducible overexpression of a mutant version lacking the tail in insect stage procyclics results in cells with an aberrant karyotype and disturbed morphology. The role of TbECK1 in the bloodstream form of trypanosomes has not been determined because neither RNA interference nor inducible overexpression worked. Interestingly, despite several attempts, a null mutant for the *L. mexicana* homolog of TbECK1 (LmxMPK6) could not be generated, suggesting this kinase is essential at least in promastigotes (John von Freyend and Wiese, unpublished data). Further analyses are required to assess its suitability as a drug target. Homozygous *L. mexicana* deletion mutants for LmxMPK11, LmxMPK12, and LmxMPK13 have been found to have no significant effect on the potential of the parasites to cause disease in Balb/c mice

(Windelberg, Scholz, and Wiese, unpublished data). TbMPK4, the *T. brucei* homolog of LmxMPK12, is not essential for any of the parasite's life cycle stages in the mammal or the insect [27]. However, procyclic stage null mutants were sensitive to increased cell culture temperatures suggesting a role of the kinase in the stress response. Finally, LmxMPK8, LmxMPK14, and LmxMPK15 have not been studied yet.

So far, other members of the MAP kinase signaling pathway in kinetoplastids have only been studied in *Leishmania*. The inability to generate a null mutant of a MAPKKK gene in *L. major* promastigotes suggests an essential role for the kinase, at least in this life cycle stage [28]. Moreover, two of the seven putative leishmanial MAPKKs have been studied in some detail in *L. mexicana*. Null mutant promastigotes for LmxMKK displayed short flagella and delayed lesion development in Balb/c mice [29]. However, the observed effect is most likely due to a delay in differentiation because LmxMKK is not detectable in the amastigote stage and therefore is unlikely to be a suitable drug target. Null mutants for LmxPK4, a MAPKK, were still able to cause lesions in infected Balb/c mice but the onset of lesion development varied considerably. Parasites isolated from lesions developing at different time points were used to infect naïve Balb/c mice and found to cause lesions with a consistently early onset. This implies that these parasites had adapted to the loss of LmxPK4 and the adaptation had occurred at different time points during the primary infection. Only once the parasite had compensated for loss of LmxPK4 could lesions develop, implying an important role for the enzyme in lesion development [30]. Again the kinase is undetectable in amastigotes and therefore might be involved in the efficiency of differentiation of promastigotes to amastigotes.

To summarize, of the 15 MAP kinases identified in *Leishmania*, three appear to be suitable drug targets as they are required for amastigote proliferation (MPK1, MPK2, MPK4). One (MPK5) is involved in disease development; however, null mutant parasites persist in the infected host. Three need further investigation to prove their role in amastigotes (MPK6, MPK7, MPK10). Five have no influence on mammalian infectivity (MPK3, MPK9, MPK11, MPK12, MPK13); and the remaining three (MPK8, MPK14, MPK15) have not been studied so far. In *T. brucei* KFR1 is currently the best candidate drug target, albeit direct evidence for its role in the host is still missing. It is followed by TbMAPK5, whose inhibition might reduce parasite numbers in the host. TbECK1 needs further investigation and TbMAPK2 and TbMAPK4 are unsuitable. No MAP kinases have been studied in *T. cruzi* to date.

Cyclin-Dependent Kinases

Another family of serine/threonine protein kinases that have been identified in kinetoplastid parasites are the cyclin-dependent kinases (CDKs). CDKs are ubiquitous in eukaryotes and play pivotal roles in the cell division cycle, acting at various "checkpoints" to prevent premature or inappropriate progression. Their activity is controlled post-translationally in a complex fashion (Figure 13.3): multiple mechan-

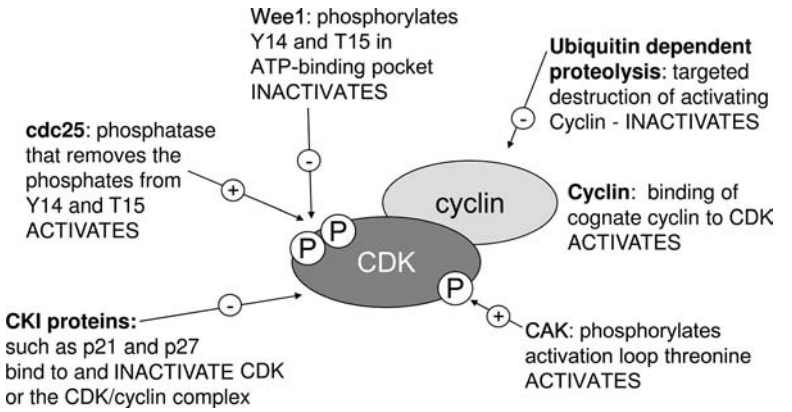


Figure 13.3 Post-translational control of CDK activity. In mammals and yeast, CDK activity is controled by multiple mechanisms: (i) binding of the cognate cyclin partner; (ii) phosphorylation of the activation loop threonine residue by the CDK-activating kinase (CAK), which increases kinase activity; (iii) phosphorylation by the dual-specificity kinase, wee1, which inhibits kinase activity; (iv) dephosphorylation by the cdc25 phosphatase, which reverses this inhibition; (v) binding of a CDK inhibitor, such as $p21^{kip}$ or $p16^{ink}$; (vi) ubiquitin-dependent proteolysis of the cyclin protein via the proteasome, which abolishes CDK activity. Although some components (cyclins, wee1, cdc25, proteasome) [9, 10, 49, 98, 99] of this regulatory machinery have been identified in trypanosomes and *Leishmania*, two important components have yet to be identified: the kinetoplastid CAK and CDK inhibitors.

isms are in place to ensure that CDKs are only activated at the correct time and for the correct duration during the cell division cycle. Control is exerted through both binding to regulatory proteins and phosphorylation [31]. Binding of the cognate cyclin partner is a prerequisite for activity and the cyclical expression and degradation of the cyclin is what primarily dictates the timing of CDK activity throughout the cell cycle [32, 33]. Phosphorylation of the CDK can result in activation or inhibition of kinase activity: phosphorylation of tyrosine and threonine in the active site by wee1 kinase prevents ATP binding and hence inactivates the CDK [34]. Conversely, phosphorylation of a threonine residue in the activation loop by the CDK activating kinase (CAK) results in vastly increased kinase activity [33, 35].

The development of pharmacological inhibitors of CDKs and their potential applications have mushroomed in recent years (reviewed in Ref. [36]), including the suggestion that they could be used to treat parasitic disease. The trypanosomatid CDKs are only approximately 50% homologous to mammalian CDKs and possess a number of divergent features, including N- and C-terminal extensions and large insert domains [37, 38]. Moreover, they have been shown to differ significantly in their sensitivity to various pharmacological inhibitors [38–40], implying that it should be possible to design or develop parasite-selective inhibitors.

The trypanosomatid parasites each have a comparatively large CDK family: 11 CDK homologs in both *L. major* and *T. brucei* and 10 in *T. cruzi* [9]. Although not all of these homologs have been shown to be *bona fide* CDKs or to play a role in cell cycle control, there are plausible reasons why these protozoa might require a relatively expanded

CDK family. The parasites possess complex cell cycles; in addition to coordinating the replication and division of nuclear DNA with cytokinesis, they must also coordinate the duplication and segregation of their essential organelles: their single mitochondrium, kinetoplast, basal body, and flagellum. Division of their highly polarized cells must necessarily be more complex than the cell division of most eukaryotic cells [41]. Thus, the complexity of their cell cycle and its integration with the life cycle, where these parasites oscillate between proliferative and cell cycle-arrested forms, may explain the comparatively large CDK family in trypanosomatid parasites.

Relatively few of the CDK homologs identified in trypanosomatids have been studied in any detail. In *Leishmania*, only LmCRK1 (cdc2-related kinase 1) and LmCRK3 from *L.mexicana* have been investigated [42, 43]. Both proteins are constitutively expressed in all life cycle stages but their activity is differentially regulated: LmCRK1 is only active in promastigotes [44], whereas LmCRK3 is active in the proliferative life cycle stages but not in the cell cycle-arrested metacyclic promastigote [38]. Both genes are essential, as indicated by the inability to create null mutants and the dramatic change in ploidy the parasites undergo to avoid loss of the essential CDK [42, 43]. The function of LmCRK1 is unknown but LmCRK3 appears to regulate progression through mitosis and its inhibition results in cell cycle arrest in the G2/M phase [43]. More recently, cyclin homologs have also been identified in the *L. major* genome, implying that the regulation of the protozoal CDKs is similar to other eukaryotes. Indeed, one cyclin homolog from *L. donovani* (LdCYC1) has been shown to bind LdCRK3 *in vitro* and coimmunoprecipitates with LdCRK3 and a histone H1 kinase activity from leishmanial lysates [45].

In *T. cruzi*, only TcCRK1 and TcCRK3 have been studied in detail. TcCRK1 is expressed in all life cycle stages [46] and is constitutively active throughout the cell cycle [40]. TcCRK1 is distributed through the cell but is especially concentrated in the kinetoplast, implying it may play a role in the regulation of this organelle [40]. Interestingly, recombinant TcCRK1 is active in its monomeric form [46] and is insensitive to CDK inhibitors, such as flavopiridol and olomoucine (Table 13.1) [40]. TcCRK1 can interact with several mammalian cyclins (E > D3 > A) [46] and was used in yeast-2-hybrid experiments to identify three *T. cruzi* cyclins, two of which also interact with TcCRK3 [40]. TcCRK3 is also expressed in all life cycle stages and its activity is cell cycle-regulated, peaking at the G2/M boundary [47]. In contrast to TcCRK1, TcCRK3 can be inhibited by CDK inhibitors; and exposure of epimastigotes to flavopiridol (Table 13.1) resulted in inhibition of parasite replication [47]. In addition, TcCRK3 can phosphorylate histone H1 *in vivo* in a cell cycle-dependent manner, again reinforcing the parallels with mammalian CDKs [48].

In addition to 11 CDKs, 10 putative cyclin homologs have been identified in *T. brucei* [49], again indicating the conservation of regulatory mechanisms in trypanosomatids. However, the cognate cyclin partner for each CDK has not yet been elucidated. CRK3 is known to interact with both a G1 cyclin, CYC2, [50] and a mitotic cyclin, CYC6 [51]. Moreover, CYC2 (also known as CycE1) has also been shown to interact with CRK1 and CRK2 [52] *in vitro*. In *T. brucei*, RNAi has enabled researchers to circumvent the difficulties of working with essential genes and selectively ablate CDKs and cyclins. Of the 11 CDKs, five have been subjected to RNAi, individually and in pairwise combinations. When ablated individually, only TbCRK1 and TbCRK3 appear

to have any effect on the trypanosome cell cycle: TbCRK1 plays a role in the regulation of the G1/S transition and TbCRK3 functions at the G2/M boundary [53]. RNAi of TbCRK4 also significantly reduced cell growth but had no apparent effect on cell cycle distribution [53]. The phenotype of *T. brucei* subjected to RNAi of TbCRK3 was very similar to that obtained for CYC6 [51] and provided additional evidence that the CRK3:CYC6 pairing occurs *in vivo*. When *T. brucei* CRKs were then subjected to RNAi in pairwise combinations, it was found that ablation of TbCRK1 and TbCRK2 resulted in a more pronounced G1 arrest in procyclic trypanosomes than that observed with RNAi of TbCRK1 alone, implying that these two CDKs function together to regulate passage across the G1/S boundary [54]. These procyclics also developed an aberrantly elongated morphology with a small proportion of cells displaying a branched posterior end [55], suggesting that these CDKs may regulate microtubule extension in the posterior end and coordinate cell growth with cell cycle progression. This phenotype is reminiscent of (but not identical to) that observed when the cyclin CYC2 was ablated by RNAi [56]; and CYC2 (also known as CycE1) has subsequently been shown to interact with both TbCRK1 and TbCRK2 *in vitro* [52]. RNAi of TbCRK1 with CycE1 and TbCRK2 with CycE1 have been performed and used to differentiate the roles of the two CDKs: TbCRK1:CycE1 appears to principally regulate the G1/S transition and TbCRK2:CycE1 primarily controls the posterior end morphogenesis [52].

As discussed above, CRK3 has been consistently shown to function at the G2/M boundary in all three trypanosomatid parasites. Moreover, CRK3 is the only trypanosomatid CDK to be formally investigated as a drug target. *L. mexicana* CRK3 was used to screen a chemical library of anti-mitotic agents, many of which are inhibitors of mammalian CDKs [39]. From 634 compounds, 27 potent inhibitors of LmCRK3 were identified, of which 16 were active against *Leishmania*-infected macrophages *in vitro*. Unfortunately, all these inhibitors also inhibit CDK1/cyclin B and present work is focused on modifying the most potent of these, the indirubins (e.g., see Table 13.1), to generate compounds which selectively inhibit the parasite CDK and have more potent antileishmanial activity [57]. CRK3 is highly conserved between the three trypanosomatid parasites (70–80% identity), opening up the possibility of developing a CRK3 inhibitor with activity against all of these medically important tropical diseases.

In summary, trypanosomatid CDKs, notably CRK1 and CRK3, are essential, function in cell cycle control, and differ from mammalian homologs in terms of their sensitivity to pharmacological inhibitors. Moreover, both *Leishmania* and *T. cruzi* proliferation in culture can be inhibited with CDK inhibitors. The future lies in the development of parasite-selective CDK inhibitors and *in vivo* screening against animal models of infection.

Casein Kinase 1

Casein kinase 1 (CK1) enzymes are a family of second messenger-independent, serine/threonine protein kinases [58]. Analysis of the genomes of *L. major, T. brucei*,

and *T. cruzi* identified CK1 homologs (6, 4, 7, respectively) in all three kinetoplastid parasites [9]. In addition a closely related tau-tubulin binding kinase (TTBK) was also identified for each parasite [9]. CK1s are highly conserved throughout eukaryotic organisms and fulfil a variety of widely differing functions [59].

Mammalian CK1s play a prominent role in the formation of membrane vesicles, especially at the synaptic cleft, where they are expressed on the surface of the vesicles and interact with other synaptic proteins [60], including those responsible for vesicle coat assembly [61]. CK1s also interact with Golgi-associated proteins, such as snapin, to regulate intracellular vesicular transport [62]. In *S. cerevisiae* the YCK1 and YCK2 proteins are known to be responsible for correct bud formation [63], a process heavily dependent on the vesicular transport of components required for polarized growth [64].

The flagellar pocket of the trypanosomatids is the exclusive site of endocytosis and exocytosis [65]. The most important aspect of this is the formation of membranous vesicles which facilitate the passage of their contents into and out of the cell body [66]. One of the best known examples of this process is the constant recycling of the *T. brucei* bloodstream form variable surface glycoprotein (VSG) coat [67]. Bound antibodies on the VSG coat are packaged into endocytotic vesicles, internalized via the flagellar pocket, and degraded, which prevents triggering of the host's complement pathway [68]. Given the prominent role of CK1s in mammalian vesicle formation, it seems likely that trypanosomatid CK1s regulate this crucial transport across the flagellar pocket.

In *T. brucei*, the flagellum is not only the organelle responsible for movement but also plays a vital role in the cell cycle. The position of the flagellar attachment zone dictates the position of the cleavage furrow [69] and maintenance of both flagellar length and motility are essential for successful completion of cytokinesis [70, 71]. In the model flagellate organism, *Chlamydomonas reinhardtii*, CK1 is physically associated with the axoneme and regulates inner arm dynein and hence flagellar movement [72]. Trypanosome CK1 may regulate flagellar movement and consequently the ability of the parasite to complete cytokinesis.

Thus, extrapolation from other eukaryotes suggests that CK1s are involved in crucial pathways in trypanosomes and therefore make attractive drug targets. Indeed recent work demonstrated that CK1 is the intracellular target for two families of kinase inhibitor which potently inhibit the growth of *L. major* promastigotes and *T. brucei* bloodstream forms in culture [73]. Analysis of the phenotype of parasites exposed to these CK1 inhibitors should shed some light on the role(s) of CK1 in trypanosomatids. Moreover, evidence suggests that parasite CK1s are significantly different from mammalian CK1s, which may make design of parasite-selective CK1 inhibitors more feasible. CK1 enzymes from several protozoa, including *T. cruzi* and *L. mexicana*, were isolated from cell lysates by affinity chromatography using an immobilized kinase inhibitor (purvalanol B) whilst no mammalian CK1s were found to bind purvalanol B under the same conditions [74]. Despite the likelihood that CK1 represents a potential drug target in protozoan parasites, there is very little published data and further work is required to fully validate these protein kinases as antiparasite drug targets.

Other Possible Protein Kinase Targets

A number of other protozoan protein kinases could also be exploited in antiparasitic drug development, including: glycogen synthase kinase-3 (GSK-3), Aurora kinases and Polo-like kinases (PLK) [75]. These families of kinases have been less extensively researched in kinetoplastids but appear to hold promise as novel drug targets.

GSK-3

Mammalian glycogen synthase kinase-3 (GSK-3) functions in many diverse signaling pathways, including insulin signal transduction, gene transcription, and regulation of the cytoskeleton (reviewed in Ref. [76]); and potential uses for GSK-3 inhibitors include diabetes and neurodegenerative diseases, such as Alzheimer's [77]. The number of GSK-3 inhibitors has burgeoned in recent years, developing extremely potent and increasingly specific inhibitors which show promise in animal models of these diseases [77].

Homologs of GSK-3 kinases have been identified in trypanosomatids [9] but very little is known about their function. In the model flagellate organism *Chlamydomonas*, GSK-3 regulates flagellar length [78]. Initial experiments indicate that GSK-3β in *Leishmania* also regulates flagellar length. Lithium inhibits *L. mexicana* promastigote growth in culture with a concomitant alteration in flagellar length (Bleicher and Wiese, unpublished data) and inhibits recombinant LmxGSK-3β *in vitro*. If GSK-3 plays a similar role in trypanosomes, it may also regulate cytokinesis since the flagellum plays a crucial role in the cell division cycle of *T. brucei* [70]. Preliminary experiments indicate that *T. brucei* GSK-3 may prove a useful drug target: RNAi of both orthologs (α, β) results in growth inhibition and inhibitors of TbGSK-3, identified through chemical library screening, inhibit bloodstream form replication in culture (F. Buckner, personal communication). Further work is required to verify that GSK-3 is the intracellular target and to develop parasite-selective compounds that do not inhibit mammalian GSK-3.

Aurora Kinase

Aurora kinases (AUK) are key regulators of mitosis, involved in chromosome condensation, mitotic spindle formation, and the spindle checkpoint itself (reviewed in Ref. [79]). An interest in AUK inhibitors as anticancer agents stems from the fact that Aurora kinases are over-expressed in many different cancer cell lines (reviewed in Ref. [80]). Several AUK inhibitors have been developed to curb tumor growth *in vivo* [81, 82]. Nondividing cells are not susceptible to AUK inhibitors, resulting in selective killing of tumor cells [82], an argument which would also hold true of parasitic infections. As yet, no clinical trials of AUK inhibitors have been reported.

Aurora kinases have been identified in all three kinetoplastid parasites [9, 83–85] but they have only been investigated in *T. brucei*. RNAi demonstrated that only one of the three *T. brucei* AUK homologs is essential [83, 85]. Ablation of TbAUK1 arrests the procyclic cell cycle prior to nuclear mitosis, with enlarged nuclei and no mitotic spindle, and also inhibits cell division [83]. Since cytokinesis can occur in the absence of mitosis in *T. brucei* procyclics [51, 86], this implies that TbAUK1 plays a regulatory role in both mitosis and cytokinesis.

Preliminary experiments to explore the potential of Aurora kinases as novel drug targets in *T. brucei* have also been performed (Larry Ruben, personal communication). TbAUK1 is essential for parasite survival in the mouse model of infection: RNAi *in vivo* within the mouse limited the infection. One Aurora kinase inhibitor, hesperadin, has been shown to inhibit trypanosome growth in the bloodstream form in culture ($IC_{50} = 50nM$) and the phenotype of trypanosomes exposed to this compound matches the TbAUK1 RNAi phenotype, implying that TbAUK1 is the (main) intracellular target (Larry Ruben, personal communication). These preliminary experiments validate TbAUK1 as a novel drug target and suggest that AUK inhibitors developed for the treatment of cancer could also be used to treat human African trypanosomiasis or be used as lead compounds in the development of parasite-selective inhibitors.

Polo Kinase

Polo kinase (PLK) serves a number of functions in mammalian cells during both mitosis and cytokinesis (reviewed in Ref. [87]). PLK acts at both the onset and exit from mitosis to coordinate the activation of the mitotic CDK with the formation of the spindle and subsequently to indirectly inactivate the mitotic CDK, via activation of the anaphase-promoting complex (APC) and mitotic cyclin degradation [88]. In mammalian cytokinesis, PLK is also multi-functional, recruiting RhoA to the site of cell division and playing a role in contractile actin ring assembly and cleavage furrow formation [89].

In *T. brucei*, the single PLK functions solely in cytokinesis regulating basal body and kinetoplast segregation. In bloodstream form (BSF) trypanosomes, TbPLK plays an additional role in cleavage furrow ingression [90, 91]. RNAi has demonstrated that TbPLK is essential in BSF, thus validating it as a potential drug target. However, the effects of PLK inhibitors on TbPLK *in vitro* or parasites in culture have not yet been reported.

Protein Kinase Inhibitors – Opportunities and Challenges

The genuine need for new drugs to treat leishmaniasis, sleeping sickness, and Chagas' disease is not in question and academia can play a significant role in discovering and validating new drug targets. However, to make the jump from target

identification to new drug requires the input of synthetic chemists, either through partnership with academics or via collaboration with the pharmaceutical industry. Inhibitors of mammalian protein kinases are being developed within the pharmaceutical industry for many different applications: cancer, Alzheimer's, diabetes mellitus, HIV, arthritis [36, 92–95]. One of the advantages of targeting parasite protein kinases is that it should be possible to "piggy back" off the extensive work already done on mammalian kinases. For instance, access to pre-existing kinase-directed chemical libraries would allow the parasitologist to screen a vast number of compounds without the cost and time of their synthesis, meaning that synthetic chemistry can be focused on the optimization of hit compounds, aiming to design or develop a parasite-selective inhibitor. Moreover, it would be simpler to compare the relative sensitivity of parasite and host enzymes to specific inhibitors because the data for the mammalian kinase has already been collected.

The main challenge will be to develop a protein kinase inhibitor which is sufficiently selective and which inhibits the target enzyme without adversely affecting any of the host's protein kinases. Despite the homology between protozoan and mammalian kinases, there are some small but significant differences between them that can be exploited in designing selective inhibitors. Many current protein kinase inhibitors target the ATP-binding site, which is largely conserved between all kinase enzymes, and yet selective inhibitors *have* been developed that can discriminate between different families of mammalian kinases or indeed between closely related members of the same kinase family [96, 97], suggesting that it should be possible to discover, design, or develop inhibitors which selectively inhibit parasite kinases. Alternatively, efforts could be focused on developing inhibitors which do not compete for ATP (as described above, e.g., Gleevec). However, the main advantage of this approach (the increased specificity) also rules out the ability to "piggy back" off mammalian protein kinase inhibitor research.

In summary, targeting protein kinases in parasitic protozoa represents an attractive proposition in the future development of novel anti-parasitic drugs. These enzymes are similar enough to allow exploitation of chemical libraries directed against mammalian kinases but different enough to design selective inhibitors which could be developed as new drugs against tropical protozoan parasites.

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14
Targeting the Malaria Kinome: Discovering Kinase Inhibitors as Novel Antimalarial Agents

*Dayadevi Jirage, Susan M. Keenan, and Norman C. Waters**

Abstract

Malaria remains a deadly disease, with over two million deaths reported each year. Mortality, morbidity, and economical burden associated with malaria are predicted to escalate due to the establishment of drug resistance throughout malaria endemic regions of the world. Drug resistance has undermined current antimalarial therapeutics and therefore a dire need for novel antimalarial agents is warranted. Protein kinases have been targeted successfully for cancer chemotherapy, cardiovascular disease, and neurological disorders. A wealth of information from these efforts may be applied to develop novel antimalarial agents. The development of kinase inhibitors as antimalarial drugs is a promising approach because many malarial kinases are essential for the developmental growth of the parasite and these inhibitors may provide chemotypes that are foreign to the parasite. Although protein kinase families are highly conserved, unique structural characteristics and regulatory mechanisms provide opportunities to develop specificity into a malarial kinase drug. A growing amount of evidence has demonstrated inhibitor selectivity differences among malarial kinases and their mammalian homologs. Malarial kinases are under investigation to gain a better understanding of their regulatory role in controlling parasite metabolism and cell cycle control. This understanding should reveal unique differences and perhaps similarities that can then be exploited for drug discovery. The use of drug combinations to combat infectious disease has gained popularity due to the increase in drug resistance. A current recommendation by the World Health Organization strongly suggests that any new antimarial drug destined for public use should be deployed in the form of a drug combination. Interestingly, there has been a paradigm shift in the development of kinase inhibitors for therapeutic indications. Traditional thinking that an inhibitor must be specific for a single kinase has been overturned. Evidence now suggests that the most successful drug candidates are those that target multiple kinases relevant to a particular disease. Simultaneous inhibition of multiple malarial kinases may prove extremely efficacious and circumvent the problems associated with the evolution of rapid rates of drug resistance.

Introduction

Malaria is responsible for well over two million deaths annually and plagues many areas of the world [1]. The highest rate of morbidity associated with malaria occurs in sub-Saharan Africa in children under the age of five. In the absence of a malaria vaccine, paucity of effective antimalarial drugs, and a significant increase in drug resistance, it is expected that without immediate intervention the morbidity and mortality associated with malaria will increase. Drug discovery efforts are rapidly advancing to push new chemical entities into the malaria drug pipeline. Several approaches are employed to include empirical screening against the malaria parasites, derivatization of known antimalarial drugs, drug combinations of pre-existing antimalarial drugs, and target-based screening [2]. Empirical screening however has fallen out of favor in the pharmaceutical industry, to be replaced with more targeted approaches. It is envisioned that target-specific approaches may be successful for malaria drug discovery. As evident from the patent literature, target-based inhibitors for the treatment of malaria is a relatively new direction in malaria drug discovery and therefore, as of yet, has not introduced many novel compounds into the pipeline [3]. This is expected to change as genetic and proteomic efforts identify and validate malaria drug targets. Several enzymes are currently pursued as malaria drug targets [4]. One class of enzymes that are pursued is the protein kinases [5, 6].

Protein Kinase Drug Discovery

Protein kinases are implicated in numerous diseases and therefore they are found in many pharmaceutical drug development pipelines [7]. Criticism however plagued these efforts because it was thought that selective kinase inhibitors could not be identified. These criticisms were justified because many kinase inhibitors are competitors of ATP binding [8]. Nevertheless, efforts moved forward with painstaking attempts to develop specific kinase inhibitors. Counter-screens were developed and kinase inhibitors thought to be specific for a kinase were advanced into clinical studies. Today, we know that developing a specific inhibitor that targets the active site of only one kinase is rare. Recent studies demonstrated that many protein kinase inhibitors once thought to be specific for one or two kinases are in fact potent against numerous kinases, and in some instances, more potent against kinases that were not considered at the time of development [9–12]. Interestingly, a few kinase inhibitors entered clinical studies and were extremely effective for their respective indications with minimal side-effects [13]. This insight resulted in a drug discovery paradigm shift from inhibiting a single kinase to targeting multiple kinases with a single compound. The key to this new approach, however, is to differentiate those kinases specific for the therapeutic indication from those not associated with the disease.

Efforts to validate and pursue protein kinase targets for malaria drug discovery constitute a relatively new approach. Advancements and setbacks learned from the kinase inhibitor field can help guide malaria kinase drug discovery. One advantage provided by ongoing human kinase drug discovery efforts is the identification of inhibitor scaffolds that can be tested against malaria kinases. Thousands of compounds enter the drug discovery pipeline, and through the course of lead identification, lead optimization and preclinical transition, many compounds are dropped from consideration [14]. Those kinase inhibitors that enter clinical studies but are dropped due to poor efficacy may provide an excellent lead scaffold for the development of malarial protein kinase inhibitors [15]. An obvious approach is to test these compounds against the malaria kinase homologs, but due to the promiscuity of kinase inhibitors, these compounds should be tested against all available malaria kinases. Compound libraries can be filtered through an *in vitro* malaria growth assay to identify those kinase inhibitors that have antimalarial activity [16]. Reducing large datasets of compounds is useful since many malaria protein kinases are not formatted for HTS assays (Figure 14.1b). Additionally, kinase inhibitors discarded from drug development may not be suitable for malaria drug discovery either but may provide valuable tools in eliciting the biological role of malaria kinases. Since the parasite is not easily amenable to genetic manipulations, these inhibitors could potentially compensate for the lack of genetic tools.

Structural Considerations for Malarial Kinases

Protein kinases share a bilobal architecture consisting of an N-terminal lobe of mostly β-sheets and a C-terminal lobe dominated by α-helices [17]. The active site is situated at the interface where ATP binds across both lobes. Phosphorylation or effector protein binding induces conformational changes in the kinases which reorients the bound ATP in the proper orientation for catalysis. Significant conformational changes distinguish active and inactive states of the protein kinase [18, 19]. Domains outside the active site of kinase, including the activation loop and the αC helix, undergo significant conformational changes that affect kinase activity and inhibitor binding. Interestingly, many of these flexible regulatory domains interact with one another to ensure the structural integrity of the active state [18, 20]. Most malarial protein kinases contain these conserved domains, suggesting that conformational flexibility is indeed important for catalysis; however, many malaria kinases contain inserts within or near these domains which may provide unique regulatory mechanisms and affect inhibitor sensitivity [21, 22] (Figure 14.2).

Predicting inhibitor sensitivity from sequence data alone can be misleading. Sequence identity among protein kinases does not necessarily equate to identical inhibitor sensitivities and kinases with poor sequence identity may actually share sensitivities to a common inhibitor [11, 12, 23]. Additionally, the constraints and molecular dynamics of the chemotype delineate the selectivity between related or

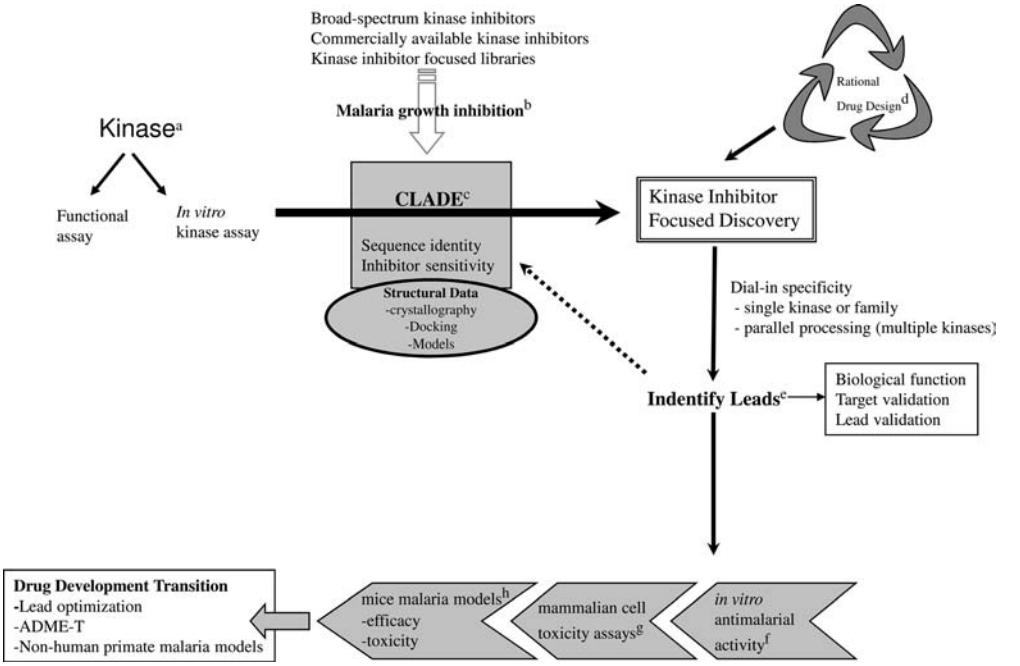

Figure 14.1 Scheme for malaria protein kinase drug discovery. (a) Kinases are developed into an *in vitro* kinase assay or a functional assay to screen inhibitory compounds. Functional assays are an option for kinases that cannot be obtained as active recombinant enzymes. However, these assays depend on an understanding of the biological role of the particular kinase. (b) Large chemical libraries are filtered through malaria growth inhibition assays to select those compounds for testing against the various kinases. (c) Kinases are grouped according to inhibitor sensitivities and sequence identity. As structural data becomes available, SAR analysis can be added to the clade. Grouping kinases in this manner facilitates parallel processing of multiple kinase targets. (d) Information from the clade drives rational drug design. An iterative process of medicinal chemistry, computational chemistry and HTS identifies a lead inhibitor. Target hopping may occur at this stage. (e) Lead compounds are valuable tools for target validation and biological function of the respective kinases. Cross-reactivity and IC_{50} data is fed into the original malaria kinase clade to expand the inhibitor profile of the kinome. (f) Lead compounds are tested against drug sensitive and drug resistance parasites. Attempts to induce drug resistance *in vitro* may be considered. (g) Mammalian cell based toxicity assays provide a counter-screen for moving compounds forward into animal models. (h) Discrepancies may arise due to differences between rodent and human malaria species since most lead compounds are developed against *P. falciparum* kinases.

unrelated kinases [24]. Unfortunately there is a paucity of structural and inhibitor sensitivity data available for malarial kinases. Many newer applications in the kinase inhibitor field rely heavily on the structural information. This includes targeting inactive versus active states of the kinase, pseudo-ring inhibitor design, exploitation of nonconserved packing defects known as dehydrons and small non ATP-binding pockets within the active site [25–29]. A better understanding of the malarial kinase space will identify unique features that may be exploited to dial-in inhibitor selectivity and target multiple kinases [30].

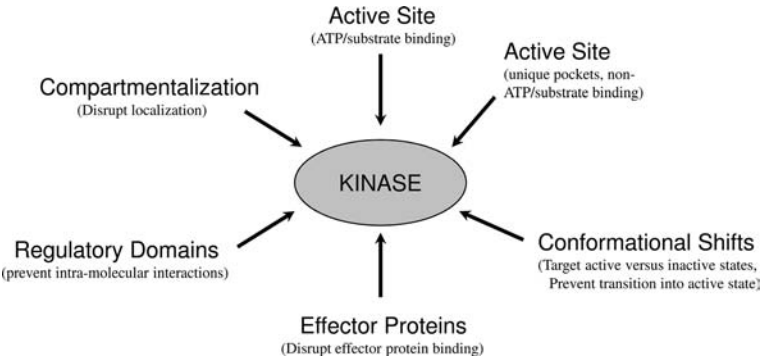

Figure 14.2 Multiple approaches to inhibit protein kinases.

Drug Resistance

Malaria drug resistance must be addressed during drug discovery [31]. Data is not available to suggest that kinases play a role in drug resistance, however a correlation exists between rapid growth and drug resistance [32]. Since many of the protein kinases are thought to be involved in signaling pathways that regulate growth, mutations that alter the regulation of the kinase may directly or indirectly affect drug sensitivity. Different strains of *Plasmodium falciparum* grow at different rates *in vitro* and it remains to be determined whether protein kinases play a role in these growth differences. Another consideration is the propensity of malarial kinases to acquire mutations that confer resistance to developed inhibitors. It has been demonstrated that a single amino acid change within the active site of a kinase can reduce the sensitivity to inhibitors. In a clinical setting, drug resistance has been documented for the cancer therapeutics gefitinib, erlotinib, and imatinib, due to single amino acid mutations in the targeted kinases [33]. Appreciatively, kinase inhibitors can be developed into drugs, however efficacy can be diminished with the advent of a single mutation. Drug resistance due to a point mutation predicts rapid resistance once a drug is deployed. Resistance to practically all antimalarial drugs is evident and in some cases resistance develops in a relatively short period of time [34]. To reduce the development of drug resistance, a drug combination strategy should be pursued for malarial kinase inhibitors; alternatively, a drug could be developed that inhibits multiple kinases.

Targeting Multiple Malarial Kinases Using Chemogenomics

Multiple malarial inhibited by a single compound may be an effective approach to increase efficacy and hinder the development of drug resistance. Classifying

malarial kinases according to inhibitory sensitivities is essential to identify cross-reactive chemotypes [11, 12]. Unfortunately there is limited malaria kinase inhibitor data; however, ongoing interest in targeting malaria kinases should result in an expansion of inhibitory profiles. As more inhibitor data becomes available, malarial kinases may be grouped into clades according to selectivity, mirroring the classification efforts pursued for human kinase drug targets (Figure 14.1c). Large panels of active kinases are required to adequately assess inhibitor selectivity. *P. falciparum* has 62–99 protein kinases (depending on which algorithms are used to identify the protein kinases from the malarial genome) [21, 22]. Kinases grouped in this manner will identify chemotypes that may inhibit multiple malarial kinases and thus facilitate parallel processing and target hopping of lead inhibitory scaffolds [24, 25]. Target hopping is an excellent approach to develop multikinase inhibitors and can reduce the cost and time associated with developing antimalarial drugs. As a starting point, commercially available kinase inhibitors, especially those that are classified as broad-spectrum kinase inhibitors should be tested against all available malarial protein kinases. This would provide the preliminary data to sort kinases according to their respective inhibitor sensitivities (Figure 14.1c). Generation of a structure–activity relationship (SAR) that correlates the malarial kinase space and the chemotype requires a focused kinase inhibitor library rich in diverse inhibitor scaffolds [35]. An iterative drug discovery process can potentially identify second and third generation inhibitor scaffolds (Figure 14.1d). During this process, scaffolds previously reported as human kinase inhibitors will probably be identified. This should not discourage malarial kinase inhibitor drug discovery efforts. Inhibitory scaffolds that are tolerated by human cells should be pursued as malarial kinase inhibitors.

Considerations for Malaria Kinase Drug Discovery

There are several considerations that should be addressed when pursuing malarial kinases as drug targets. A protein kinase should be amenable to preliminary characterization before pursuant as a drug target, either as *in vitro* kinase assays using recombinant proteins or novel cell-based functional assays using parasite or parasite extracts (Figure 14.1a; Table 14.1). Target validation is a bottleneck in drug discovery and remains a challenging endeavor with limited genetic and biochemical tools available to the malaria drug discoverer [36]. In an effort to streamline the process, concurrent target validation and lead validation can occur (Figure 14.1e; Table 14.1). Once an inhibitor is identified, pharmacological validation can be achieved. A weakness of this approach however is that the selected inhibitor may have antimalarial activity through off-target effects. Chemogenomic or genetic knockdown/knockout approaches would be more definitive; however these approaches may not be amendable to all protein kinases. In addition to determining the essentiality of a protein kinase, the parasite's ability to tolerate inhibition of the pathway which the kinase regulates should be evaluated. It has been demonstrated

Table 14.1 Coupling target and lead validation for malaria kinase drug discovery.

Target Validation	Outcome	Lead Identification	Outcome
Gene knockout/ knockdown	Not essential	Parasite sensitive to inhibitor	Off-target
	Not essential	Parasite sensitivity to inhibitor decreases	Multiple kinase targets
Chemical genetics[a]	Kinase essential	Parasite insensitive to inhibitor[b]	On-target
Overexpress kinase[c] (strong/weak promoter)	Measurable phenotype	Parasite sensitivity to inhibitor decreases	On-target
In vitro growth inhibition (pharmacological validation)	Kinase inhibitor kills parasites	Induce inhibitor-resistant mutations in kinase target[d]	On-target
		Functional assay	

[a]Chemical genetic approaches are a viable option if the kinase cannot be knocked out due to essentiality. Transfected parasites carrying an inhibitor sensitive or inhibitor insensitive kinase mutant can be used for target and lead validation.
[b]Inhibitor sensitivity is compared to genetically unmodified parasites of the same strain.
[c]Caution should be used to interpret overexpression studies until the expression levels between wild type and transgenic strains are defined. These studies should account for any differences in sensitivity of the kinase regulated pathway.
[d]Inhibitor resistant mutant kinases should be validated using recombinant kinases via *in vitro* inhibition kinase assays and by transfection of wild type parasites with the inhibitor- insensitive kinase mutant.

that weak kinase inhibitors may be more effective than potent ones because a cell may not tolerate perturbation of the pathway due to full or partial inhibition of the pathway-associated kinase [37]. In this regard, a correlation between a functional assay and antimalarial activity should be established.

Many malarial kinase inhibitors will cross-react with human kinases and therefore counter-screens must be deployed to select compounds specific for malarial kinases. An obvious counter-screen is one that utilizes the human homolog of the malaria kinase. Due to the promiscuity of kinase inhibitors, these screens are limited since many inhibitors cross-react with multiple kinases from different and distinct families [11, 12]. Even for those "orphan" malarial protein kinases that do not phylogenetically cluster with any eukaryotic protein kinase, appropriate counter-screens must be deployed [21]. Mammalian cell-based assays may provide the most useful information regarding toxicity of compounds (Figure 14.1g). The challenge however is to select mammalian cell types that are most informative regarding kinase inhibitor sensitivity and toxicity. The cell lines selected should be suitable for determining an *in vitro* therapeutic index against the malaria parasite. Indexes may be more meaningful than a toxicity IC_{50} threshold because toxicity may depend on which kinases are inhibited, how long they are inhibited and differences between full or partial inhibition. There is growing evidence to support analysis and correlation

between *in vitro* kinase inhibition and cell-based physiological response to the inhibitors [38]. As more correlative data between kinase inhibition and cell toxicity emerges from human protein kinase drug discovery efforts, the malarial protein kinase efforts will benefit.

Finally, the stage specificity of the kinase should be considered. A kinase that only functions at a specific phase of the parasite cycle may not be an ideal target as a kinase that is essential for the entire development of the parasite. Additionally, inhibiting a kinase that is part of a kinase activation cascade may actually amplify the inhibitory action of a selected inhibitor. To date, kinase cascades and signaling transduction pathways in general have not been fully explored or identified in the parasite. As these pathways are identified, it is expected that some kinase targets will be dropped and others pursued. These considerations may not only identify valid protein kinase targets, but also be used to rank malarial protein kinases on their potential as malaria drug targets and therefore prioritize which protein kinases should be pursued.

Malarial Protein Kinases

Several malarial protein kinases are pursued as drug targets and it is expected that the number will increase as additional kinases are characterized from the malaria parasite. Here we discuss those malarial protein kinases that are the best characterized as potential antimalarial drug targets. Representatives from each of the major classes of kinase families are presented (Table 14.2).

GMGC Kinase Family

Pfmap-1

Pfmap-1 [PF14_0294] is a member of the MAPK family [39]. Clustering analysis recently suggested that Pfmap-1 is preferentially related to ERK8 [40] rather than the classic ERK1/2, P38 or JNK subfamilies. As ERK7/8 are not activated in the typical three-component system [21], Pfmap-1 membership in this subfamily of kinases seems appropriate, considering the lack of MAPKKs (or indeed MAPKKKs) identified in the *Plasmodium* genome. Pfmap-1 is expressed in both asexual parasites and gametocytes [41–43]. Reverse genetic experiments have demonstrated that Pfmap-1 is not essential for schizogony or gametocytogenesis in intraerythrocytic infection, nor for gametogenesis or sporogony in the mosquito vector [44]. Interestingly, protein levels of Pfmap-2 are increased in Pfmap-1 negative *P. falciparum* clones, suggesting that Pfmap-2 may compensate for the activities of Pfmap-1.

Pfmap-2

Pfmap-2 [PF11_0147] was cloned in the course of attempting to identify additional members of the three-component MAPK signaling cascade following the isolation of

Table 14.2 Characteristics of potential malarial kinase drug targets.

Kinase	PlasmoDB ID	Cellular Processes[a]	Peak mRNA expression[b]	Structural data	Sequence identity to nearest human homolog	Unique inserts or amino acids that may affect regulation or inhibitor sensitivity	Inhibitors	References
CMGC								
PfGSK3	PFC0525c	ND	Late trophozoite, schizont, gametocyte, sporozoite	Homology model (GSK-3β)	GSK3-beta	ND	Indirubin-3-monoxime, flavopiridol Alsterpaullone, Gwennpaullone Hymenialdisine	[69]
PfPK1, GSK-related	PF08_0044	ND	Early ring and merozoites	ND	GSK3-beta/alpha	ND	ND	[21]
GSK3-related kinase (putative)	MAL13P1.84	ND	Late ring, gametocytes	ND	GSK3-alpha	ND	ND	[21]
Pfmap-1	PF14_0294	ND	Late trophozoite, schizont, gametocyte, sporozoite	ND	MAPK15	ND	ND	[39]
Pfmap-2	PF11_0147	Asexual propagation	Late ring, gametocytes, sporozoite	ND	MAPK1	Nontraditional TSH activation motif	ND	[45]
PFPK6	PF13_0206	ND	Trophozoite, schizont	Homology model (CDK2)	CDK2	SKCILRE and the TXY; Nontraditional TPT activation motif	Roscovitine	[47, 49]

(*Continued*)

Table 14.2 (*Continued*)

Kinase	PlasmoDB ID	Cellular Processes[a]	Peak mRNA expression[b]	Structural data	Sequence identity to nearest human homolog	Unique inserts or amino acids that may affect regulation or inhibitor sensitivity	Inhibitors	References
Pfcrk-5	MAL6P1.271	ND	Ring, schizont	ND	PCTAIRE protein kinase 1	ND	ND	[21]
Pfmrk	PF10_0141	ND	Ring, trophozoite, early schizont	Homology model (CDK2, CDK7)	CDK7	Two unique inserts Unique active site residues Autophosphorylation	WR216174 Kenpaullone Purvalanol A, I ndirubin-3, mono-xime, oxindoles, 3-phenyl-quinoli-nones, isoquinoline sulfonamides, chalcones and tryptanthrins	[54–58]
PfPK5	MAL13P1.279	DNA replication	Trophozoite; early schizont, gametocyte; sporozoite	1V0P; 1V0O; 1V0B, 1OB3	CDK1-3	Ability to adopt active conformation in response to inhibitor association Autophosphorylation	Purvalanol A Purvalanol B, olomoucine, flavopiridol, indirubin-3 monoxime, xestoquinone	[62, 65, 72]

Pfcrk-1	PFD0865c	Development	throughout	ND	P58, CDK11	Unique 370AA charged amino acid rich N terminal region, AMTSLRE motif	ND	[66]
NIMA								
Pfnek-1	PFL1370W	Signal transduction	throughout	ND	NIMA related kinase 2	conserved NIMA kinase FXXT motif is replaced with a MAP/ERK related SMAHS	Xestoquinone	[71, 72]
Pfnek-3	PFL0080c	Signal transduction	gametocytes	ND	NIMA related kinase 9	ND	ND	[73]
AGC								
PfPKA-c	PFI1685w	Parasite survival	Schizont	Homology model (PKA)	PKA-C	Residues within, around, and at the periphery of the PKI-binding site in mouse PKA-C have been replaced in PfPKA-c. Two amino acid changes within the H-89 binding site of porcine PKA-Cα subunit: M120 and Y122 to L112 and F114 in the plasmodial sequence	PKI, H89	[75]

(*Continued*)

Table 14.2 (*Continued*)

Kinase	PlasmoDB ID	Cellular Processes[a]	Peak mRNA expression[b]	Structural data	Sequence identity to nearest human homolog	Unique inserts or amino acids that may affect regulation or inhibitor sensitivity	Inhibitors	References
PfPKA-R	PFL1110c	Modulating anion channel activity in the membranes of *P. falciparum*-infected red blood cells	Higher in asexual stages compared to the sexual stages	Homology model (PKA)	PKA-RI and RII	The single PKA-R subunit in *P. falciparum* shares characteristics with both mammalian RI and RII subunits	PKI	[76]
PfPKB	PFL2250c	Along with Ca^{2+}/CaM involved in merozoites' invasion of RBCs	Schizont and merozoite	Homology model PKB	PKB/Akt	Lacks the PH domain Autophosphorylates	A443654 PKC-specific inhibitors Go 6976 and Go 6983	[82–84]
PfPKG	PF14_0346	Sexual development – specifically exflagellation of male gametocytes	Ring and gametocyte	Homology model PKG	PKG	Contains extra cGMP-binding motifs	(Compound 1)	[80, 81]
CAMK								
PfCDPK1	PFB0815w	Merozoite invasion and membrane biogenesis in parasite	Intraerythrocytic stages, membrane fraction of ring stages	Arabidopsis CDPK	CDPKs not identified in vertebrates	PfCDPKs contain a basic cluster and acylation sites as membrane-anchoring motifs	ND	[86–88]

PfCDPK2	PFF0520w	ND	All erythrocytic stages except segmentor	ND	ND	ND	ND	[89]
PfCDPK3	PFC0420w	ND	Sexual stages	ND	ND	ND	ND	[21]
PfCDPK4	PF07_0072	ND	Male gametocyte	ND	ND	ND	ND	[21]
Casein Kinase								
PfCK1	PF11_0377	ND	mRNA levels maximum in early rings, kinase activity maximum in late trophozoite	Human CK2α	CK1	Smallest among the reported CK enzymes, lacks autophosphorylation activity and C-terminal inhibitory regions like other CKs	Purvalanols, Isoquinolinesulfonamides, CK1-7 (Compound 2)	[95, 97]
Orphan Kinase								
PfPK9	PF13_0085	Ubiquitin mediated signalling pathways	Early trophozoite	ND	SNF-1	Autophosphorylation at T082, T265, and T269. Putative forkhead-associated domain (FHA)	ND	[103]
PfPK7	PFB0605w	Signaling pathways, Parasite growth and development	Trophozoite/ early schizont, gametocytes	2PMO; 2PMN; 2PML	MAPK3	Four inserts Tyrosine gatekeeper residue	Staurosporine, Hymenialdisin, K510, K109, K497,1NM-PP1, PP2	[100]

ND: Not determined.
[a]Cellular process based on available data.
[b]Peak mRNA Expression from data within Plasmodb (www.plasmodb.org).

Pfmap-1 [45] however, Pfmap-2 was classified as a MAPK rather than as a member of the MAPKK (MEK) subfamily. Interestingly, Pfmap-2 does not possess the characteristic TXY activation motif associated with MAPK activation and conserved in Pfmap-1; the substituted TSH motif suggests a unique mode of regulation for this kinase. Phylogenetic analysis of the malarial kinome did not cluster Pfmap-2 with the typical MAPKs [21]. Recently, work by Dorin-Semblat *et al.* [44] demonstrated that Pfmap-2 is essential for asexual propagation at the stage of erythrocytic schizogony while its role in gametocytogenesis and indeed gametogenesis (as suggested in *P. berghei*) remains under investigation [46].

PfPK6

PfPK6 [PF13_0206] incorporates features associated with both MAPK and CDKs and this premise was corroborated by recent phylogentic analysis [21]. Deviations of standard motifs include the absence of a canonical PSTAIRE domain associated with CDK association with cyclin (substituted with SKCILRE) and the TXY activation motif associated with MAPKs is changed to TPT [47]. Autophosphorylation by PfPK6 has been demonstrated *in vitro* but PfPK6 is not inhibited by $p21^{CIP1}$ CDK inhibitor [48]. Weak inhibition of kinase activity has been demonstrated with roscovitine (IC_{50} 30 μM) and olomoucine (IC_{50} 180 μM). Inhibition of CDK2 activity in response to roscovitine (IC_{50} 0.7 μM) is greater, while ERK1 and ERK2 inhibition are similar (IC_{50} 30 and 14 μM, respectively) which suggests a PfPK6 inhibition profile with greater similarity to ERK than CDKs. Homology models docked with low-affinity inhibitors are available [49]. Collectively, the biochemical and inhibition data of PfPK6 suggest functionality similar to MAPKs than to CDKs. The hybrid nature of PfPK6 makes it an interesting target for further analysis.

Pfmrk

Pfmrk [PF10_0141] was identified as a putative CDK with greatest similarity to CDK7 [50]. Similarity with CDK7, the CDK-activating kinase (CAK), includes increased activity when complexed with cyclin H and in the presence of Pfcyc-1 (a Pf cyclin with maximal homology to cyclin H), as well as increased activation in the presence of PfMAT-1 (a homolog of the mammalian assembly factor, MAT1 [51–53]). Unlike CDK7 however, Pfmrk has not been shown to activate other kinases, specifically the CDK1 homolog, PfPK5 [52]. Pfmrk autophosphorylation has been shown, as has inhibition by $p21^{CIP1}$ [48, 51]. While collectively these data suggest a functional similarity with CDKs, recent phylogenetic analysis shows Pfmrk midway between the CDK and MAPK clusters [21]. A number of compounds from a series of oxindoles [54], 3-phenyl-quinolinone [55], isoquinoline sulfonamides, [56] and tryptanyhrins [57, 58] have been identified as Pfmrk inhibitors. Pfmrk has two unique inserts: a small five amino acid insert prior to the cyclin binding motif and a larger insert within the activation loop. While there is no crystallographic information available for Pfmrk, homology models based on an active conformation of CDK2 [51, 59] and inactive conformation of CDK7 [56] have been validated and provide insights into the role of another structural modification that could play a role in inhibitor sensitivity. Phe143 is located within the ATP-binding site and provides π–π stacking

interactions for inhibitor stability, but it also effectively decreases the overall size of the binding site. Mutational analysis of the active site identified residues important for catalysis and inhibitor binding [53].

PfPK5

PfPK5 [MAL13P1.279] shares sequence identity with CDK1 and CDK2, has been shown to have activity against casein and histone H1 [60], and is suggested to play a role in the regulation of DNA replication [61]. High-resolution structures of PfPK5 are available: an apo structure, in association with known kinase inhibitors purvalanol B and indirubin-5-sulfonate, and incorporating a T198A point mutation to explore the CDK1-like phosphorylation site [62]. PfPK5 adopts the characteristic kinase bilobate fold and is structurally similar to the inactive form of CDK2. PfPK5 associates with malarial cyclins (Pfcyc-1, -2, -4), mammalian cyclins (p25, cyclin H, cyclin A3), and a noncyclin related activator of mammalian CDK1/2, RINGO; and, once bound to cyclin, it is able to autophosphorylate [62–64]. Interestingly, PfPK5 is not a substrate for known CAKs (CDK7/cyclin H; human or *Xenopus laevis*) and Cak1p (*Saccharomyces cerevisiae*) which correlates with independence of phosphorylation within the activation loop as a prerequisite for activation [62]. PfPK5 activity is inhibited by known kinase inhibitors [62, 65]. High similarity in composition of binding site residues with CDK1 and -2 suggests the ability to "piggy back" on the active research for inhibitors of these mammalian kinases in order to identify potent PfPK5 inhibitors but also suggests selectivity of compounds for PfPK5 and against host CDKs may be difficult to achieve. It is interesting that while PfPK5 (in association with cyclin A) is sensitive to inhibition by Purvalanol B and NU6102, it is insensitive to the larger compounds indurbin-5-sulfonate and staurosporine. CDK2/cyclin A is sensitive to all four compounds [62] and both kinases are insensitive to indirubin-3′-monoximine. Indeed, all inhibitors examined that have affinity for PfPK5 have higher IC_{50} values against the malarial kinase than against CDK2; compounds with the opposite selectivity would be preferable as they would provide a proof of principle for selectivity. The available structures of PfPK5 with bound inhibitors do however offer insights into possible mechanisms by which selectivity can be dialed-in for PfPK5. Flexibility in the glycine-rich region of PfPK5 and the overall ability of the active site to rearrange conformationally in response to inhibitor association is far greater for PfPK5 than CDK2. In addition, sequence differences adjacent to the ATP active site (a His substitution for Phe, an Asp substitution for Ser/His) can be exploited to achieve selectivity. Similar sequence differences have been exploited successfully to achieve inhibition selectivity between the closely related mammalian kinases CDK1/2 and CDK4/6. The promiscuity of PfPK5 activation by cyclins can also be explained by structural properties that enable the kinases to very easily adopt an active conformation. The proposed role in DNA replication and the availability of structural information support PfPK5 as a potential drug target.

Pfcrk-1

Pfcrk-1 [PFD0865c] is a cdc2-related kinase with apparent similarity to Pfcrk-2, -3, -4, and -5 [21]. Based on the stage-specific accumulation of mRNA in gametocytes, Pfcrk-

1 appears to be developmentally regulated [66]. The kinase has maximal sequence homology to the $p58^{GTA}$ family of kinases, but to date, no *in vitro* activity has been demonstrated and therefore functional similarity to $p58^{GTA}$ has not yet been shown. Sequence variations compared to other protein kinases include a Ser at position 381 which is a variant for the expected Gly and an AMTSLRE motif. The N-terminal region also contains a unique 370 charged amino acid-rich extension [66]. Crk-2 has also been identified from *P. knowlesi* and *P. berghei* and is expressed at low levels throughout the asexual cycle, in the mosquito and during gametogenesis [67]. Crk-2 from *P. vivax* can compensate for a temperature-sensitive *Schizosaccharomyces pombe* cdc2 mutant and induce cell cycle arrest [68], providing evidence that cdc-related kinases play a role is cell cycle regulation.

PfGSK3

PfGSK3 [PFC0525c] is homologous to the mammalian GSK-3β [69]. Mammalian GSKs constitute a highly conserved family of kinases that play numerous physiological roles, including Wnt and insulin signaling, glycogen synthesis, cell proliferation and adhesion, apoptosis, and embryogenesis. PfGSK3 is able to phosphorylate known GSK3 substrates, including glycogen synthase, axin, and Tau, but is not able to bind to axin. Intracellular localization studies have shown that the kinase is expressed in early trophozoite and exported into the RBC cytoplasm. Kinase inhibitors such as staurosporine, indirubin-3′-monoximine, alesterpaullone, and hymenialdisine are high-affinity inhibitors of PfGSK ($IC_{50} > 0.05\,\mu M$). Structural information gleaned from a homology model of PfPGSK3, coupled with the availability of enzymatic inhibition studies, indicates PfGSK-3 as a potential drug target [69]. However, known toxicity and negative side-effects of GSK3 inhibitors examined in numerous disease states (including Alzheimer's disease and diabetes) suggest potential limitations for this target [70].

NIMA Kinase Family

Pfnek-1

Pfnek-1 [PFL1370W] is a never in mitosis, *Aspergillus* (NIMA)/NIMA-like related kinase. The expected conserved NIMA kinase FXXT motif is replaced with a MAP/ERK related SMAHS in Pfnek-1. The second serine of the SMAHS motif was identified as essential for *in vitro* activation [71]. The FXXT motif contains the target residue for a necessary activational phosphorylation event for NIMA/NIMA-like kinases. The apparent dichotomy of identity between NIMA kinase (sequentially) and MAPK (activation mode) suggests Pfnek-1 as a potentially druggable target. Interestingly, Pfnek-1 is able to phosphorylate the atypical MAPK Pfmap-2 and these kinases show significant activational synergy in the *in vitro* phosphorylation of mylein basic protein. Pfnek-1 is not however able to phosphorylate the traditional MAPK, ERK1 *in vitro*. Laurent *et al*. identified xestoquinone, a molecule isolated from a Vanuatu marine sponge as an inhibitor of Pfnek-1. Xestoquinone is also a weak inhibitor PfPK5 but does not inhibit PfPK7 or PfGSK-3. *In vitro* antiplasmodial

activity and weak *in vivo* activity against *P. berghei* NK65-infected mice has been shown, although the *in vivo* activity is negatively impacted by toxicity [72].

Pfnek-3

Pfnek-3 [PFL0080c] is able to phosphorylate and activate Pfmap-2 *in vitro* (as for Pfnek-1) suggesting MAPKK-like activity [73]. Mutational analysis of the atypical TSH motif on Pfmap-2 suggests that T290 is essential for the activation of Pfmap-2 by Pfnek-3 [74]. With no identified traditional MAPKK (MEK) homologs in the parasitic genome, the ability of Pfnek-1 and -3 to enhance the activation of Pfmap-2 suggests that the NIMA kinases are MAPKK-like and as such are potential targets for drug discovery.

AGC Kinase Family

PfPKA-c

PfPKA-c [PFI1685w] has been characterized [75]. PfPKa-c contains all 15 signature amino acid residues conserved in serine/threonine protein kinases. However, some nonconservative amino acid substitutions within the catalytic sites of PfPKA-c have been identified that could potentially contribute to specificity of the enzyme [75]. In their study, Syin *et al.* [75] demonstrated that the PfPKA-c mRNA expression is upregulated in the asexual schizont stages and downregulated in the sexual stages of the parasite's life cycle. PfPKA-c associated kinase activity is highest in intraerythrocytic schizonts. This activity could be inhibited by both PKI and H-89. Furthermore, the addition of cell-permeable H-89 to cultures resulted in arrest of parasite growth, suggesting that PfPKA-c may perhaps be essential for parasite viability. Two amino acid residues within the H-89 binding site of porcine PKA-Cα subunit were demonstrated to be substituted in the plasmodial sequence and may contribute to inhibitor specificity. Screening derivatives of H-89 against recombinant PfPKA-c could lead to a compound specifically targeting the kinase and hence the parasite [75].

PfPKA-R

PfPKA-R [PFL1110c] was recently characterized [76]. PfPKA-R contains two cyclic nucleotide-binding site signatures called phosphate-binding cassettes that specifically identify the R subunits of PKAs. Interestingly, this single PfPKA-R shares structural characteristics with mammalian RI and RII subunits. PfPKA-R mRNA expression is higher in asexual stages compared to the sexual stages of parasite growth. Parasites overexpressing PfPKA-R displayed a growth defect that could be rescued by increasing the intracellular cAMP levels. Addition of either the exogenous recombinant PfPKA-R protein to the patch clamp experiment pipette or overexpressing PfPKA-R in parasites led to the downregulation of anion conductance. This demonstration of the effect of the cAMP-dependent pathway on the anion channel activity in the membranes of *P. falciparum*-infected RBCs has provided new insights into the mechanisms of how this parasite modifies host cell infiltration pathways. This protein target could now be explored for antimalarial drug discovery [76].

PfPKG

PfPKG [PF14_0346] has been characterized from *P. falciparum* [77]. Structural analysis of the PfPKG gene revealed the presence of three cGMP-binding motifs and a fourth degenerate motif. Mutational analyses of the four cGMP-binding sites revealed that the third site has maximal effect on enzyme activity and that the fourth site is required for maximal PfPKG activity [78]. Addition of cGMP stimulated the phosphotransferase activity of a truncated recombinant form of PfPKG ($PfPKG_2$). Analogs of cGMP were tested for effect on the *in vitro* phosphotransferase activity of $PfPKG_2$. Interestingly, the membrane-permeable analog 8-pCPT-cGMP which activates mammalian PKG more strongly than cGMP itself [77] did not significantly stimulate $PfPKG_2$ activity. This result suggests possible structural differences within the cGMP binding site of PfPKG and could be explored for possible therapeutic interventions. PfPKG is predominantly detected in rings and gametocytes stages [79]. The cGMP signaling pathway has been implicated in sexual development of *P. falciparum* as guanylyl cyclase activity is present in mature gametocyte membranes and is stimulated by xanthurenic acid – a mosquito-derived activator of exflagellation [80, 81]. Addition of the protein kinase inhibitors H-8, H-89 and staurosporine to gametocytes inhibited the process of exflagellation (an important step in male gametogenesis), suggesting a role for PfPKG in gamete formation. Additionally, a trisubstituted pyrrole 4-[2-(4-fluorophenyl-5-(1-methylpiperidine-4-yl)-1H pyrrol-3-yl] pyridine referred to as Compound 1, inhibited the *in vitro* activity of recombinant $PfPKG_2$ as well as partially purified native PfPKG. Compound 1 also demonstrated antimalarial activity against *P. falciparum* and extended survival in a *P. berghei* acute infection mouse model [79]. Detailed biochemical and cell biological studies are needed to establish the mechanism of action of Compound 1 and to establish a definitive role for PfPKG in the parasite's life cycle.

PfPKB

PfPKB [PFL2250c] has been identified and characterized [82]. Interestingly, PfPKB shares a high level of sequence similarity to the catalytic domains of both mammalian PKB (71%) and PKC (64% to all three isoforms α, β, γ). Since a plasmodial PKC homolog has not yet been identified, PfPKB has been suggested to be its closest relative in *P. falciparum*. Recent studies have demonstrated that the structural organization and mechanisms of PfPKB regulation are markedly different from its mammalian counterpart [82–84]. The most striking structural feature of PfPKB is that it lacks a phosphoinositide interaction pleckstrin homology domain (PH domain). Biochemical studies also demonstrate that autophosphorylation is a prerequisite for its activity unlike its mammalian counterpart that is activated by PDK1 and that the protein's N-terminal domain plays a regulatory role. The N-terminal domain prevents the activation of PfPKB by inhibiting its ability to autophosphorylate [82]. PfPKB is expressed in schizonts/merozoites. Two isoform-specific inhibitors of PKC, Go 6983 and Go 6976 were tested against PfPKB in an *in vitro* kinase assay. Go 6983 inhibited PfPKB activity with an IC_{50} value to $\sim 1\,\mu M$, while Go 6976 failed to inhibit PfPKB. Treatment of parasite cultures with Go 6983 resulted in decreased parasitemia after the late schizont/segmentor stage (40–44 h),

specifically resulting in a decrease in the number of rings by about 60%. Based on these results, a role for PfPKB in schizont-to-ring transition was proposed. Surprisingly, the Ca-binding protein calmodulin (CaM) was identified as an upstream regulator of PfPKB both *in vitro* and *in vivo* [83]. Treatment of parasites with phospholipase C inhibitor U73122 decreased PfPKB activity and reduced the amount of CaM associated with PfPKB. Specific CaM inhibitor W7 also inhibited ring formation and invasion of RBCs by free merozoites. The authors proposed a model wherein Phospholipase C mediates release of Ca from intracellular stores, leading to PfPKB activation by CaM. A component of the glideosome, PfGAP45, was identified as a substrate of PfPKB [84]. GAP45 is known to play a role in anchoring the actin-myosin motor complex to the Inner Membrane Complex. Therefore, phosphorylation of a component of this complex by Ca-dependent signaling pathways was proposed to be an important step in the invasion process. The PKB inhibitor, A443654, was found to inhibit PfPKB activity, prevent the formation of rings and inhibit invasion by merozoites. Similar results were obtained with peptide inhibitors designed on the CaM-binding motif of the N-terminal region of PfPKB [84].

Calcium-Dependent Protein Kinase Family

PfCDPK1

PfCDPK1 [PFB0815w] activity was demonstrated in membrane fractions [85], and subsequently, several CDPKs were identified [21] and characterized: PfCDPK2 [PFF0520w], PfCDPK3 [PFC0420w], and PfCDPK4 [PF07_0072] [86–91]). PfCDPK1 [86] and -2 [89] were demonstrated to be prototypical CDPKs, with the recombinant proteins displaying Ca-dependent kinase activity. PfCDPK1 was shown to phosphorylate proteins of the host erythrocyte membrane [86]. Recent studies have revealed that PfCDPK1 is myristoylated both *in vivo* and *in vitro*, which plays a role in membrane anchoring of the protein [85]. The protein was detected in the parasitophorous vacuole and the tubovesicular system of the parasite. Based on signature motifs present in PfCDPK1, its post-translational modifications and subcellular localization, the authors presented a model in which PfCDPK1 plays a role in membrane biogenesis in the parasite [85]. PfCDPK3 was shown to be specifically expressed in gametocytes, suggesting a role for this gene in linking Ca^{2+} to the process of gametogenesis [90]. The PbCDPK3 gene product in *P. berghei* was conclusively shown to play a role in ookinete gliding motility [92]. The *Pbcdpk3*$^{-}$ parasites produced morphologically normal ookinetes that failed to engage the mosquito midgut epithelium due to marked reduction in their gliding ability. This resulted in a reduction in malaria transmission to the mosquito [92, 93]. These studies suggest that PbCDPK3 is a potential drug target for preventing parasite motility and blocking malarial transmission to the mosquito vector. Pharmacological studies performed on *P. gallinaceum* also suggest that calcium and calmodulin antagonists block zygote to ookinete differentiation [94]. PbCDPK4 was shown to regulate XA-induced gamete formation and subsequent mosquito transmission in

P. berghei [91]. Using transgenic *P. berghei* strains expressing a GFP–aequorin Ca^{2+} sensor, the authors demonstrated that PbCDPK4 is a putatitive Ca^{2+} effector in the gametocyte. Studies performed on PbCDPK4 knockout strains suggest that PbCDPK4 functions as a specific regulator of cell cycle progression in the XA-activated gametocyte and is required for ookinete infectivity in the mosquito midgut epithelium [91].

Casein Kinase Family

PfCK1

PfCK1 [PF11_0377] has been characterized [95]. Its corresponding CK1 activity has also been purified from parasite extracts, using affinity chromatography with the isoquinolinesulfonamide compound CK1-7. PfCK1 mRNA is maximally expressed in early ring stage, while interestingly the PfCK1 activity is highest in trophozoites. PfCK1 is unique because it is the smallest reported CK enzyme and lacks autophosphorylation activity and C-terminal inhibitory regions reminiscent of other CKs. Malarial casein kinase activity was reported to phosphorylate the erythrocyte 4.1 protein [96]. CK enzymes are susceptible to purvalanol compounds *in vitro* and inhibitors of CKs have also demonstrated antiparasitic activity [97–99]. The *in vivo* function and promise of PfCK1 as a drug target warrants further investigation.

Orphan Protein Kinases

PfPK7

PfPK7 [PFB0605w] is referred to as an orphan kinase as it is not readily classified into a eukaryotic protein kinase family [21]. There is limited sequence similarity to MAPK3 and serine-threonine kinase 6 (AURORA-A kinase) [100], however biochemical analysis failed to demonstrate MEK activity. PfPK7 is able to autophosphorylate and to phosphorylate the generic substrates myelin basic protein, histone H2A, and casein [101]. Reverse genetic studies recently indicated a dual-stage role for the kinase. In the asexual growth phase, PfPK7 negative parasitic clones exhibit a decrease rate of growth and release fewer merozoites per schizont rupture [102]. Also, the ability to produce oocytes in the mosquito vector is significantly reduced. Potential ATP competitive inhibitors have been sought for PfPK7 and a series of known PKA and MAPKK(MEK) inhibitors was initially analyzed with limited success (no inhibitors identified with $IC_{50} > 30\,\mu M$) [101]. A later screen of >500 compounds identified the closely related chemical family members K510, K109, and K497 (also 1NM-PP1 and PP2, both known Src kinase inhibitors) as PfPK7 inhibitors. Structural coordinates for PfPK7 are available in an active conformation in association with K510 and hymeniadisine. Based on structural similarity, PfPK7 is most similar to the MAPKKK, TAO2 [100]. PfPK7 has four inserts, most notably a 17-residue insert (I2) starting at Glu 63 and a 19-residue insert (I4) beginning at Asp 270.

PfPK7 is constitutively active when purified from *E. coli*, and for activation, phosphorylation within the activation loop is not required. Interestingly, the expected phosphorylatable threonine/serine or negatively charged residue within the activation loop usually necessary for rearrangement of this loop during activation is replaced in PfPK7 with an Arg. The positively charged Arg does appear to be compensated for by other negatively charged residues within the loop, allowing the overall mechanism of kinase activation to be maintained. There are several structural features that could be exploited for PfPK7 drug design. First, the gatekeeper residue is a tyrosine. The identity of the gatekeeper residue controls access to a hydrophobic pocket within the ATP-binding site. The structural coordinates suggest that this pocket is accessible in PfPK7. Also affecting the size and accessible space within the binding site is the addition of an amino acid (Asp 123) in the hinge region. This insertion, which is also observed in AURORA-A, decreases the overall size of the binding pocket and affects drug design [100]. Collectively, the lack of a mammalian homolog, a role in asexual replication, and the availability of crystallographic data to aid in drug design suggest PfPK7 as a potential drug target.

PfPK9

PfPK9 (UBC13 kinase) [PF13_0085] does not classify into any eukaryotic kinase family and only shares 11% identity with the closest eukaryotic protein kinase, SNF-1 [103]. PfPK9 is expressed throughout the asexual stage of development and peaks in schizonts. Interestingly, PfPK9 is localized at the parasitophorous vacuolar membrane (PVM) during late rings but with the parasite plasma membrane in schizonts. The particular localization on membranes may constitute a receptor-like kinase signaling pathway. Localization may also provide a regulatory mechanism to control kinase activity or substrate specificity. Robust *in vitro* kinase activity has been demonstrated for PfPK9 which appears to be regulated through an autophosphorylation mechanism [103]. The ubiquitin-conjugating enzyme, PfUBC13, is an endogenous substrate of PfPK9. UBC13 is thought to play a role in nonproteolytic signaling pathways and its activity appears to be regulated through PfPK9 phosphorylation. Although the essentiality of PfPK9 has not been explored, the characteristics of PfPK9 suggest that it may be a good drug target due to its possible role as a receptor-like kinase and a regulator of ubiquitin signal pathways and its unique sequence/structural characteristics that can be exploited.

Conclusion

We present an overview of specific members of the malarial kinome. The malarial kinome offers a number of interesting targets for antimalarial chemotherapeutics, and with rapid advances in high-throughput screening, techniques of molecular genetic manipulation and structural biology, the coming years will hopefully herald a new era in malaria drug discovery.

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15
Malaria and Antimalarials – a Focused View

*Frank Seeber**

Abstract

Human tropical malaria caused by the protozoan parasite *Plasmodium falciparum* is one of the three big infectious diseases, with high mortality rates each year. Due to resistance development against safe and affordable antimalarials, the search for new drug targets and inhibitors thereof that could be developed into new chemotherapeutics is still a major goal of many research groups worldwide. A great number of potential targets have been identified through *in silico* analysis of the parasite's known genome sequence. In this chapter I focus on a few metabolic pathways that are known to be localized in a plastid-like organelle (apicoplast) and have been shown to be essential for parasite survival. I present their inhibition by preclinical or experimental drug-like substances as well as some understudied auxiliary steps. Finally, I briefly discuss some recent developments that might lead to new perspectives for finding new drug targets.

Introduction

In a book published in 1955 entitled "*Man's mastery of malaria*," the author expressed his concerns that, with the eradication of malaria around the corner, not only would the *Plasmodium* parasite causing this deadly disease vanish from earth but that also many malariologists would be without any future work to do [1]. The reasons for such a belief at this time were the widespread use of the insecticide DTT (seemingly able to eradicate the vector mosquitoes responsible for the transmission of *Plasmodium* to the human host) and chloroquine (CQ) to kill the parasite. Now, more than 50 years later, funding for research on vaccine and drug development to fight malaria is at its peak, reflecting the fact that the causative agent of human malaria is still with us.

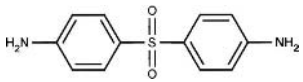
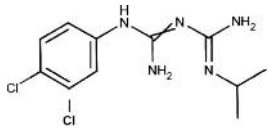
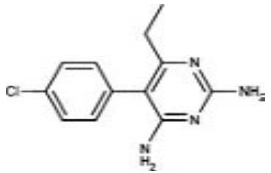
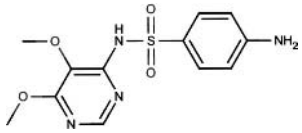
Human malaria is caused by several species of the intracellular apicomplexan parasite *Plasmodium* spp., with *P. falciparum* being the most widespread and also the most dangerous species, responsible for tropical malaria. According to the most recent world malaria report, 3.2 billion people are affected by the disease worldwide, of whom more than a million die each year, mostly young children and pregnant women [2]. In more than 100 countries people are at risk of contracting the disease. The main reason for this steady increase during the past 25 years lies in the fact that in many developing countries support for primary health services has declined, sometimes combined with an interruption of eradication measures [2]. In addition, in countries where AIDS and malaria co-occur, an increased incidence of severe malaria in adults has been observed, due to a generally lower cell-mediated immunity [3].

However, the most important factor responsible for the still serious worldwide malaria situation is the ongoing development of drug resistance in both the vector [4] and the parasite population [5]. The 1960s saw the first reports indicating that the most successful drug against malaria, the 4-aminoquinoline chloroquine (CQ; Table 15.1) was no longer working in some areas due to resistant *P. falciparum* populations [6]. Today, 80–100% of field isolates are resistant to CQ. This is particularly sad because in general CQ is a safe, effective, and cheap drug, three premises a chemotherapeutic should have if the aim is to eradicate the disease in developing countries [7].

Thus, in 1998 the Roll Back Malaria (RBM) initiative was initiated by the WHO, the World Bank, UNICEF, and the United Nations Development Program. Its aim was to reduce the global malaria burden to 50% by the year 2010 and to halt its incidence in 2015. Although this goal might not be achieved within this time-frame, the prerequisites to do so in the near future are at hand. The genome of the host *Homo sapiens* as well as that of the vector *Anopheles gambiae* and those of several species of *Plasmodium* are now known [8, 9]. *Inter alia*, this enables researchers to compare metabolic pathways between pathogen and host and to draw conclusions as to which potential drug targets might be the most promising to focus on. Further, finding the target of a proven drug with the help of genomics and proteomics (or any other "omics") is also of value when combination therapies (CT) have to be devised with drugs affecting different targets [10]. CT is now a promising approach to treat malaria in areas with widespread resistance to monotherapy [5, 11].

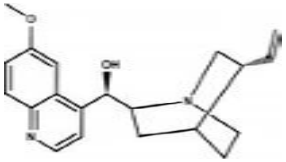
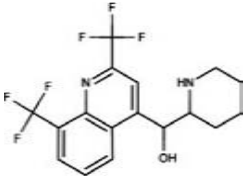
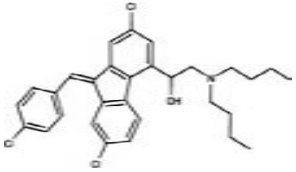
PubMed, when queried with the terms "malaria" and "chemotherapy", lists more than 2200 articles from the past five years and more than a hundred that deal with new drug targets (accessed April 2008). Therefore, it is impossible to condense this information into a short chapter, and a subjective selection on the topic was made. After giving a very brief overview of the drugs currently in clinical use, I focus on the plastid-like organelle of *Plasmodium* that has been recognized in recent years to contain a number of unique and essential metabolic pathways, some of which have already been exploited as drug targets (Figure 15.1). I then finish with a short discussion on recent strategies and approaches that should allow us to define the next generation of antimalarials.

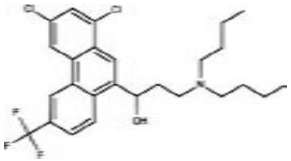
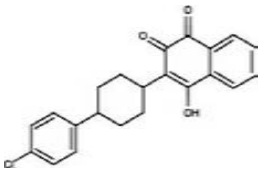
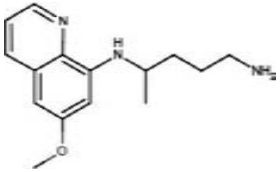
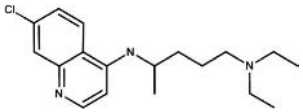
Table 15.1 Drugs and drug combinations currently in use for treatment and/or prophylaxis of malaria.

Name structure	Drug class[a] (Acc. No. at DrugBank or KEGG Drug) chemical name	Used in CT[b] (trade name)	Indication[c]	Mode of action	Res.[d]	IC_{50} *in vitro* (nM)[e]
Dapsone 	Antifolate (DB00250) 4-(4-aminophenyl) sulfonylaniline	+ Chlorproguanil (LapDap) ⇒ withdrawn!	Acute Pf malaria; prophylaxis	Dihydropteroate synthase inhibitor	−/+	30
(Chlor-) Proguanil[f] 	Antifolate (APRD00188) 1-[amino-[(4-chlorophenyl) amino]methylidene]-2-propan-2-yl guanidine	+ Atovaquone (Malarone)	Acute Pf and Pv malaria; prophylaxis	DHFR inhibitor	++	5–40
Pyrimethamine 	Antifolate (APRD00599) 5-(4-chlorophenyl)-6-ethylpyrimidine-2,4-diamine	+ Sulfadoxine/ Artesunate	Acute mild Pf malaria; prophylaxis	DHFR inhibitor	++	10–30
Sulfadoxine 	Antifolate (DB01299) 4-Amino-N-(5,6-dimethoxy-4-pyrimidinyl) benzenesulfonamide	+ Pyrimethamine/ Artesunate	Acute mild Pf malaria	Dihydropteroate synthase inhibitor	++	290–900

(*Continued*)

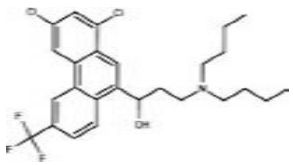
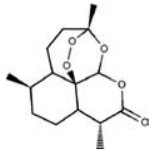
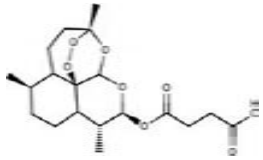
Table 15.1 (*Continued*)

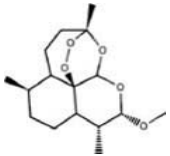
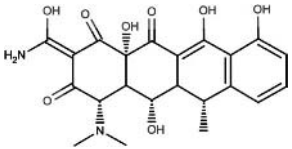
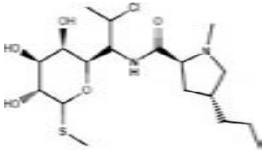
Name structure	Drug class[a] (Acc. No. at DrugBank or KEGG Drug) chemical name	Used in CT[b] (trade name)	Indication[c]	Mode of action	Res.[d]	IC_{50} *in vitro* (nM)[e]
Quinine 	Arylaminoalcohol (APRD00563) (R)-[(5R,7S)-5-ethenyl-1-azabicyclo[2.2.2]octan-7-yl]-(6-methoxyquinolin-4-yl)methanol	+ Clindamycin or Doxycycline	Acute severe Pf malaria	Possibly interference with heme digestion	+	60–380
Mefloquine 	Arylaminoalcohol (APRD00300) [2,8-bis(trifluoromethyl) quinolin-4-yl]-piperidin-2-ylmethanol	+ Artesunate or Artemisinin	Mild to moderate acute Pf and Pv malaria; prophylaxis	Possibly food vacuole where it forms toxic complexes with heme	+	3–11
Lumefantrine 	Arylaminoalcohol (--/D03821) 2-(dibutylamino)-1-[(9E)-2,7-dichloro-9-[(4-chlorophenyl) methylidene] fluoren-4-yl]ethanol	+ Artemether (Co-artem: Riamet)	Acute Pf malaria; prophylaxis	Possibly interference with heme digestion	+	12–45

Halofantrine 	Arylaminoalcohol (APRD00419) 3-dibutylamino-1-[1,3-dichloro-6-(trifluoromethyl) phenanthren-9-yl]-propan-1-ol	—	Acute severe Pf malaria	Unknown; possibly food vacuole where it forms toxic complexes with heme	+	2–10
Atovaquone 	Hydroxynaphtoquinone (APRD00805) 3-[4-(4-chlorophenyl) cyclohexyl]-4-hydroxy naphthalene-1,2-dione	+ Proguanil (Malarone)	Acute Pf malaria; prophylaxis only as CT	Blocks mitochondrial electron trsp. chain	+	0.5–6
Primaquine 	8-aminoquinoline (APRD00604) N-(6-methoxyquinolin-8-yl) pentane-1,4-diamine	—	Radical cure of established infections with Pv and Po; prophylaxis	Unknown, possibly inhibition of electron transport, or oxidative stress generation		1000–1500
Chloroquine 	4-aminoquinoline (APRD00468) N′-(7-chloroquinolin-4-yl)-N, N-diethylpentane-1,4-diamine	—	Acute mild Pf malaria; prophylaxis where still possible	Prevents polymerization of heme into hemozoin	+++	4–15

(*Continued*)

Table 15.1 (*Continued*)

Name structure	Drug class[a] (Acc. No. at DrugBank or KEGG Drug) chemical name	Used in CT[b] (trade name)	Indication[c]	Mode of action	Res.[d]	IC_{50} *in vitro* (nM)[e]
Amodiaquine 	4-aminoquinoline (APRD00796) 4-[(7-chloroquinolin-4-yl)amino]-2-(diethylamino methyl)phenol	+ Artesunate or + Pyr/Sulfa	Acute mild Pf malaria	See Chloroquine	++	8–18
Artemisinin 	Artemisinin (--/D02481) (3R,5aS,6R,8aS,9R,12S,12aR)-octahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepin-10(3H)-one	+ Mefloquine	Acute Pf malaria; prophylaxis	Possibly generation of radicals and/or inhibition of Ca^{2+}-ATPase	−/+	8–21
Artesunate 	Artemisinin (--/D02482) Butanedioic acid, 1-[(3R,5aS, 6R,8aS,9R,12S,12aR)-octahydro-3,6,9-trimethyl-3,12-epoxy- 12H-pyrano [4,3-j]- 1,2-benzodioxepin-10-yl] ester	+ Amodiaquine or + Mefloquine or + Pyr/Sulfa	Acute Pf malaria; prophylaxis	See Artemisinine	−/+	0.5–3.5

Artemether 	Artemisinin (--/D02483) 3,12-Epoxy-12H pyrano[4,3-j]-1,2-benzodioxepin, decahydro-10-methoxy-3,6,9-rimethyl-(3R,5aS,6R,8aS,9R,10S,12R,12aR)-	+ Lumefantrine (Co-artem: Riamet)	Acute Pf malaria; prophylaxis	See Artemisinine	−/+	1.5–16
Doxycycline 	Antibiotic (DB00254) (2Z,4S,4aR,5S,5aR,6R,12aS)-2-(amino-hydroxymethylidene)-4-dimethylamino-5,10,11,12a-tetrahydroxy-6-methyl-4a,5,5a,6-tetrahydro-4H-tetracene-1,3,12-trione	+ Quinine	Acute mild Pf malaria; prophylaxis	Blocks apicoplast translation	+	450–2000
Clindamycin 	Antibiotic (APRD00566) (2S,4R)-N-[2-chloro-1-[(2R,3R,4S,5R,6R)-3,4,5-trihydroxy-6-methylsulfanyl oxan-2-yl]propyl]-1-methyl-4-propylpyrrolidine-2-carboxamide	+ Fosmidomycin (phase II trials) or + Quinine	Acute mild Pf malaria; CT	Blocks apicoplast translation	−/+	5–10 000; depends on time-scale of assay

Data were compiled from different literature sources [2, 5, 6, 12–15, 31, 111, 112].

[a] Accession numbers are for DrugBank entries ([113]; http://www.drugbank.ca) or, where no entry is available (--/), for KEGG Drug (http://www.genome.ad.jp/kegg/drug).

[b] Indicates whether the drug is used in combination therapy (CT). Trade names are given for some combinations.

[c] Indicates whether single drug is used in CT and names its partner(s). Pf, *P. falciparum*; Pv, *P. vivax*; Po, *P. ovale*.

[d] Res. – Resistance in the field: + + + widespread resistance; + + resistance in some areas; + isolated resistance; −/+ field resistance thought to be possible but not observed yet; — no clinical reports of resistance.

[e] Range of 50% inhibitory concentrations (IC_{50}) determined *in vitro*.

[f] Crossed double bond – "double either" – indicates cis or trans configuration.

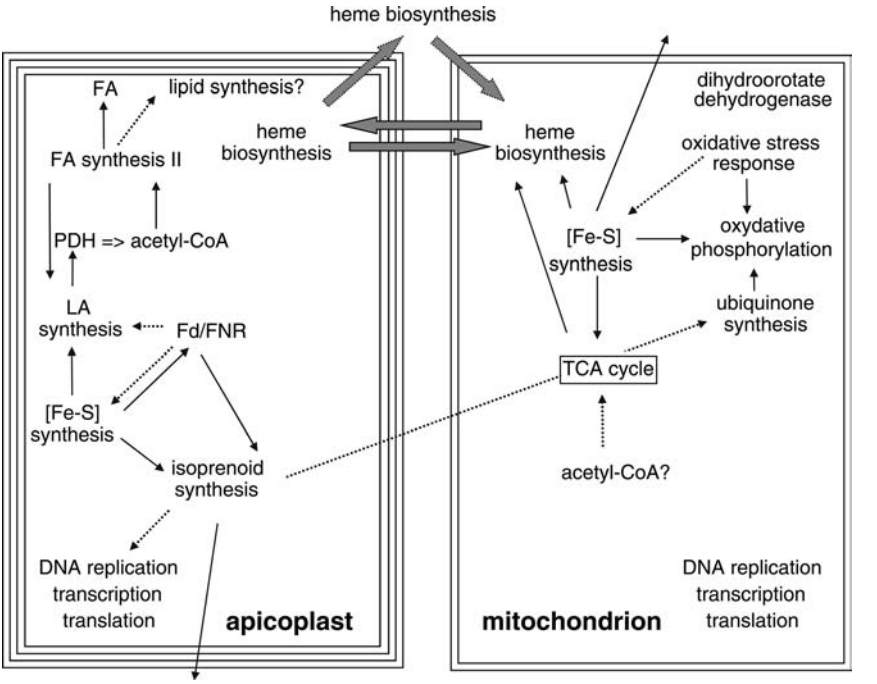


Figure 15.1 Schematic of the confirmed or presumed pathways operating in the apicoplast and mitochondrion, respectively. Dotted arrows are unconfirmed, solid arrows are experimentally verified interactions. Note that the heme pathway is most likely distributed between the two organelles and the cytosol, and that the two organelles are always closely associated *in situ*. For further information see Refs. [33, 110].

Currently Used Drugs

The drugs currently available for clinical use and which are more or less still active against the different plasmodial species are summarized in Table 15.1. For more comprehensive and detailed information on this topic and on other recently emerged drug targets and active compounds, the reader is referred to a number of recent excellent indepth reviews [12–15].

New and Emerging Drug Targets in *Plasmodium*

With the completion of the genome projects for *P. falciparum* and several other species [16], it became possible to provide a provisional map of the proteome of these parasites.

Not too surprising, more than 60% of the predicted or confirmed open reading frames result in proteins unique either to *Plasmodium* or to the phylum [16]. This is bad news if one wants to understand the biology of the organism because no direct links can be made to already known functions from related proteins of other organisms. However, it is likely good news when it comes to drug development since compounds that inhibit whatever function of these proteins are potentially less likely to cause severe side-effects in humans than if similar structures already exist in the host of the parasite (obviously, there are a number of well tolerated drugs that prove the opposite; see Table 15.1 and below). In any case, the obstacle is: (i) to show that a protein or pathway is essential for the survival of *Plasmodium* and (ii) that the protein is "druggable", that is, that small molecules can bind and interfere with the function of the protein [17]. From this short discussion it is obvious that a large number of potential drug targets should be present in the *Plasmodium* genome, and that in order to find the needle in the haystack, either high-throughput methods have to be employed, or we can use the more traditional "educated guess" approach, based on some prior knowledge of candidate metabolic pathways or cellular processes (see Ref. [7] for a recent review on drug target assessment). In this part I concentrate on the latter and give an overview of how an unanticipated biological event (secondary endosymbiosis) in apicomplexan parasites resulted in the "provision" of a number of essential metabolic pathways that are unique to the parasite and are now actively explored by several groups as promising new drug targets. Of course, a variety of other parasite-specific processes have been identified in recent years that are essential for survival of *P. falciparum* and other apicomplexans and for which inhibitors have been described or are actively developed (for these the reader is referred to some recent reviews [13, 15, 18–24]).

The Essential Plastid-Like Organelle in *Plasmodium* Contains a Number of Proven Drug Targets

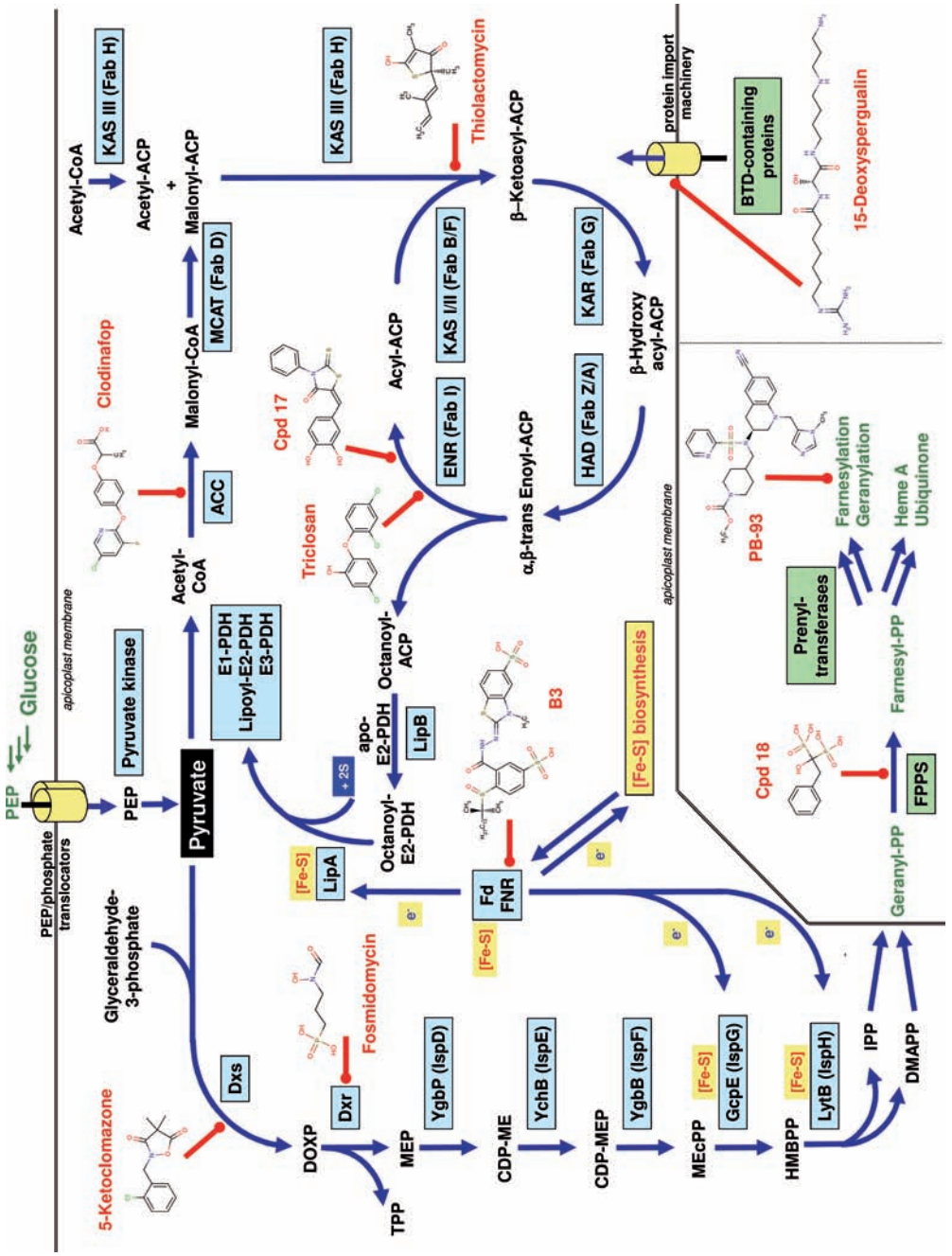
With the sequencing of a circular 35 kb extrachromosomal DNA of *P. falciparum* in 1991, it became evident that there was an evolutionary link between algae and parasites [25]. In 1996/97 two groups provided molecular evidence of the colocalization of the circular genome and an enigmatic structure described morphologically in the 1960s and 1970s [26, 27]. The organelle is now called the "apicoplast" and is thought to have arrived in an ancestor of extant Apicomplexa through the process of secondary endosymbiosis of an alga (for reviews on the biology of the apicoplast see Refs. [28, 29]). Almost all Apicomplexa seem to possess this organelle, which is basically a highly reduced plastid (both in function and in its genome content). Its presence and function is crucial for their survival, reflected by the fact that most drugs affecting plastid-localized targets are effective against many apicomplexan species (e.g., *Toxoplasma gondii*, *P. falciparum* [30]). This conservation in function is also due to considerable sequence homology in the respective proteins, making *T. gondii* a good model to study or infer *P. falciparum*'s apicoplast metabolism, since the former is

more amenable to genetic manipulation and possesses a superior morphology compared to *Plasmodium*. Therefore, I frequently refer below to findings done in this parasite.

Although the proteins required for transcription, translation, and replication are encoded on the genome of the apicoplast and are already known to be the target for several antibiotics [30, 31], the majority of proteins constituting the proteome of the apicoplast are nucleus-encoded and have to be transported to the organelle via the secretory pathway using a bipartite targeting domain (BTD) at their N-terminus [32]. This feature allowed the development of predictive computer algorithms that culminated in a list of 545 putatively apicoplast-localized proteins (about 10% of the predicted proteins in *P. falciparum*), of which >70% are of unknown function [33]. Although a number of proteins are known that do not possess a BTD (e.g., some metabolite transporters of the apicoplast membrane [32]), this structural feature is not only very helpful in assigning some functions to the apicoplast (Figure 15.1) but is in itself a potentially very interesting research object for drug developers, since the protein import machinery that recognizes the BTD is a unique feature not found in the host [32]. For example, the immunosuppressant 15-deoxyspergualin has been shown in *P. falciparum* to inhibit the trafficking of proteins destined for the apicoplast, followed by apicoplast mis-segregation during subsequent cell divisions and ultimately death of the parasite [34]. Clearly, an immunosuppressant is not a good drug candidate for malaria patients but it is a proof of principal that this cell biological pathway is of crucial importance and that small molecules can interfere with it. Studies are underway to unravel the import machinery of secondary plastids like the apicoplast and might lead to new structures and ideas how to inhibit it [35].

It became apparent very early that plant-derived metabolic pathways (due to the ancestry of the apicoplast from plastids) could be ideal candidates for new drug targets. Although not every postulated plant-derived pathway could finally be linked to the apicoplast (e.g., the shikimate pathway for the generation of chorismate [36]), three major metabolic and at least one auxiliary pathways that are encoded by nuclear genes have been shown to be essential for parasite survival and are druggable (see Figure 15.2). These are the bacterium (or dissociated)-type fatty acid biosynthesis

Figure 15.2 Metabolic pathways in the apicoplast, starting from pyruvate. The part left of pyruvate is the isoprenoid synthesis pathway, whereas the one on the right is FAS II. Fd/FNR (center) connects both pathways. Compound names and enzymes (filled boxes) drawn in green are not found within the apicoplast but in the cytosol or mitochondrion, respectively, whereas those in the apicoplast are shown in black/blue. Inhibitors and the step they target are indicated in red. Abbreviations: ACC, acetyl-CoA carboxylase; ACP, acyl carrier protein; BTD, bipartite targeting domain; CDP-ME, 4-diphosphocytidyl-2-C-methyl-D-erythritol; CDP-MEP, 4-diphosphocytidyl-2-C-methyl-D-erythritol-2-phosphate; DMAPP, dimethylallyl diphosphate; DOXP, 1-deoxy-D-xylulose-5-phosphate; Dxr, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; Dxs, 1-deoxy-D-xylulose-5-phosphate synthase; ENR, enoyl-ACP reductase; Fd, ferredoxin; FNR, ferredoxin-$NADP^+$ reductase; FPPS, farnesyl pyrophosphate synthase; HAD, beta-hydroxyacyl-ACP dehydratase; HMBPP, (E)-4-hydroxy-3-methyl-but-2-enyl diphosphate; IPP, isopentenyl diphosphate; KAR, 3-ketoacyl-ACP reductase; KAS I/II, beta-ketoacyl synthase I/II; KAS III, 3-ketoacyl-ACP synthase III; MCAT, malonyl CoA-ACP transacylase; MEcPP, 2-C-methyl-D-erythritol-2,4-cyclodiphosphate; MEP, 2-C-methyl-D-erythritol-4-phosphate; PDH, pyruvate dehydrogenase; PEP, phosphoenol pyruvate; PP, pyrophosphate; TPP, thiamine pyrophosphate.


Glucose
PEP
PEP/phosphate translocators
apicoplast membrane
Pyruvate kinase
PEP
Pyruvate
Acetyl-CoA
ACC
Malonyl-CoA
MCAT (Fab D)
Acetyl-CoA
KAS III (Fab H)
Acetyl-ACP
+
Malonyl-ACP
KAS III (Fab H)
Thiolactomycin
Clodinafop
Cpd 17
Triclosan
Acyl-ACP
ENR (Fab I)
KAS I/II (Fab B/F)
β–Ketoacyl-ACP
KAR (Fab G)
β-Hydroxy acyl-ACP
HAD (Fab Z/A)
α,β-trans Enoyl-ACP
protein import machinery
BTD-containing proteins
15-Deoxyspergualin
PB-93
Farnesylation
Geranylation
Heme A
Ubiquinone
apicoplast membrane
E1-PDH
Lipoyl-E2-PDH
E3-PDH
+ 2S
apo-E2-PDH
LipB
Octanoyl-ACP
Octanoyl-E2-PDH
B3
[Fe-S] biosynthesis
Prenyl-transferases
Cpd 18
Farnesyl-PP
FPPS
Geranyl-PP
[Fe-S]
LipA
Fd
FNR
e⁻
Glyceraldehyde-3-phosphate
Dxs
5-Ketoclomazone
DOXP
Dxr
Fosmidomycin
TPP
MEP
YgbP (IspD)
CDP-ME
YchB (IspE)
CDP-MEP
YgbB (IspF)
MEcPP
GcpE (IspG)
HMBPP
LytB (IspH)
IPP
DMAPP

(FAS II) and its dependent generation of the co-factor lipoic acid (LA), the non-mevalonate (or DOXP/MEP) isoprenoid synthesis pathway, and some steps of heme synthesis (not covered here, see Ref. [37]). They are all more or less interconnected at some point, and thus inhibition of one pathway might also affect the others to some extent (Figure 15.2). A central molecule in this respect is pyruvate, since it serves as a precursor for both the DOXP and the FAS II pathway. It derives from glycolysis-derived phosphoenolpyruvate (PEP) that is transported into the apicoplast and then converted into pyruvate by pyruvate kinase. It serves either as precursor (together with glyceraldehyde-3-phospate, GA3P) for the first step in isoprenoid biosynthesis or it is converted by the multienzyme complex pyruvate dehydrogenase (PDH) into acetyl-CoA, which is the principal building block of the growing acyl chain of fatty acids. Transport of PEP into the apicoplast depends on two membrane-resident PEP/phosphate translocators [38–40]. The proteins are known and could be useful in the design of analogs that inhibit the transporters. Given the importance of both pathways for *Plasmodium*'s survival this would seem to be a worthwhile effort.

Isoprenoid Biosynthesis

Isoprenoids constitute the biggest class of compounds in nature and are required for cell signaling, post-translational modifications of proteins and tRNA and also, for example, as precursors for ubiquinone biosynthesis [41]. In contrast to its mammalian host which synthesizes the two initial building blocks of isoprenoids, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) from mevalonate (a precursor derived from the degradation of leucine; mevalonate pathway), *Plasmodium* and other apicomplexans (as well as plants and most eubacteria) use exclusively the second pathway, starting with pyruvate and glyceraldehyde-3-phosphate and yielding DOXP (1-deoxy-D-xylulose 5-phosphate). This synthesis route is therefore called the DOXP or nonmevalonate pathway [42]. The enzymatic synthesis consists of seven steps, and the final products IPP and DMAPP are then condensated to isoprenoids of higher complexity using farnesyl pyrophosphate synthase (FPPS) as the first enzyme in these subsequent steps (Figure 15.2). FPPS is located in the mitochondrion of *T. gondii* [43], and consequently IPP/DMAPP presumably have to be transported into this organelle, as it is the case for other apicoplast metabolites (see below). Apicoplast and mitochondrion are always closely associated in *P. falciparum* and *T. gondii*, and the exchange of compounds is one likely reason for this phenomenon.

Inhibitors for three enzymes in this long cascade are known so far, and two of them inhibit the growth of *P. falciparum in vitro* and *in vivo* [44–48]. The most advanced drug is fosmidomycin (Fos), an antibiotic that inhibits the second enzyme in the pathway, DOXP reductoisomerase (Dxr), with an IC_{50} of 300–1200 nM, depending on the *Plasmodium* strain [49]. Fos has been tested in early phase II clinical trials and found to be effective against acute, uncomplicated malaria at a high cure rate and with few side-effects, but only when used in combination with clindamycin (Table 15.1),

whereas recrudescence was observed when Fos was used alone. This combination thus appears to be a promising alternative for the future. Other chemical structures have been shown to inhibit Dxr, but all with substantially higher IC_{50} values than Fos and its somewhat more potent derivative FR900098 (see Ref. [49]).

The enzyme situated before Dxr, DOXP synthase (Dxs), can be experimentally inhibited by a metabolite of the herbicidal compound clomazone, 5-ketoclomazone [47]. Although this substance has only been tested against the recombinant enzymes from plants, algae, and *Escherichia coli* and found to inhibit at fairly high concentrations (>50 μM [50]), it could provide a lead compound for more potent inhibitors of this enzyme. In contrast to Dxr inhibition, which shuts down only isoprenoid biosynthesis, blockage of Dxs function could potentially also affect another important pathway of the parasite, namely the synthesis of thiamine pyrophosphate (TPP) [51]. However, whether *P. falciparum* follows the DOXP-dependent route for TPP generation is currently not entirely clear. It should be noted that TPP is an essential co-factor for the apicoplast-resident pyruvate dehydrogenase subunit E1 and for Dxs itself, so synergism might be possible.

From this point on, the enzymes that condensate IPP and DMAPP to more complex isoprenoids are generally also present in the host. Nevertheless, several bisphosphonates are potent inhibitors of FPPS in Apicomplexa, including *P. falciparum* [44, 46]. In *T. gondii* this enzyme is bifunctional, generating geranyl pyrophosphate and farnesyl pyrophosphate from IPP and DMAPP [43]. Although bisphosphonates are used as inhibitors of bone resorption and approved for the treatment and prevention of human diseases like, for example, osteoporosis and tumor metastases in bone, they have been shown to be active in the low micromolar range against *T. gondii* and *P. falciparum* without apparent toxicity to the host cells [46]. Interestingly, a study in *E. coli* indicated synergism between one potent bisphosphonate derived from a screen and Fos [52], and it would be important to know whether similar effects can be obtained in *P. falciparum*.

Finally, moving further down the road of isoprenoid usage, several potent inhibitors of different chemical classes of protein farnesyltransferases have been described that act against *P. falciparum in vitro* and against mouse plasmodia *in vivo* [53–57]. However, the most potent inhibitor with sufficient oral availability described so far (tetrahydroquinoline-based PB-93) still would need improvement due to rapid clearance in the blood [58]. This led to the decision of the "Medicine for Malaria Venture" to end its active support for this project due to concerns about the pharmacokinetic profile [59]. This once more highlights the difficulties in transferring inhibitors that act *in vitro* with low nanomolar IC_{50} values into drugs for use in patients.

Fatty Acid and Lipoic Acid Biosynthesis

The second vital pathway that is directly dependent on apicoplast-generated pyruvate is the FAS II pathway resident in this organelle (Figure 15.2) [60, 61]. Fatty acids (FAs)

are mainly required as building blocks of lipids, as energy stores, and as precursors for other molecules, including second messengers and cofactors. Although *Plasmodium* can take up FAs from the host cell, the parasite is critically dependent on their *de novo* synthesis. This was shown first in 1998, using the β-ketoacyl ACP synthase (KAS I/II) inhibitor thiolactomycin [62]. Several other classes of compounds directed against different FAS II enzymes have been reported (for comprehensive reviews see Refs. [61, 63]). The most advanced target in terms of known active compounds, 3D structure of the enzyme and structure–activity relationships is the enoyl-ACP reductase (PfENR or PfFabI) of *P. falciparum*. It can be efficiently inhibited by the well known bactericide triclosan (IC_{50} 700 nM) and offers protection against infection with *Plasmodium* in animal models [64, 65]. Numerous triclosan derivatives and different Fab I inhibitors have been developed [66–68], with one member of rhodanines (compound 17) being of similar potency *in vitro* as triclosan [69], which still remains the "gold standard" of PfFab I inhibitors. However, better bioavailability of triclosan is desired (the compound has to traverse multiple membranes in order to act in the four-membrane-bound apicoplast) and to this end ester "prodrugs" have been designed that show better penetration and better *in vitro* activity than unesterified triclosan [70].

An important point in this respect is the recent finding that FAS II genes and some interconnected genes (see below) are significantly upregulated in the liver stage of the parasite [71, 72] and also in a subset of blood-stage *Plasmodium* taken directly from patients [73]. These data should give an additional impetus to develop FAS II inhibitors into clinical drugs, since the killing of early liver stages could also result in increased immunity due to the release of protective antigens of this stage.

However, at this point a cautionary note has to be made regarding the often repeated misconception that a bacterium-type FAS II has no counterpart in the host (e.g., Ref. [71]). It relates to the fact that lipoic acid (LA), a dithiol-containing medium-chain fatty acid, is an essential cofactor for several 2-oxo acid dehydrogenase complexes, including pyruvate dehydrogenase (PDH) and the mitochondrial 2-oxoglutarate dehydrogenase (OGDH) [74, 75]. For its synthesis a functional FAS system has to be operating in the same organelle (Figure 15.2), and since human mitochondria are no exception they consequently also contain a FAS II-like machinery [76]. Although the level of sequence identities are low between plasmodial FAS II enzymes and those of the human host (unpublished observation), their similar enzymology suggest the potential of unwanted side-effects if new FAS II inhibitors are not rigorously tested for specificity. In fact, triclosan is known to inhibit FAS in human cancer cells at moderately high concentrations *in vitro* (>10 μM; [77]).

The dependence of LA synthesis on FAS is mutual, since the already-mentioned conversion of pyruvate to acetyl-CoA absolutely requires a PDH complex that is post-translationally modified by LA (Figure 15.2) [78–80]. It follows that LA synthesis in the apicoplast, which requires two enzymes (LipA, LipB), should also be a valid drug target, although no inhibitor has been described so far. However, 3D structures of LipA/LipB from bacterial species are known and could aid in the design of specific inhibitory compounds.

The Ferredoxin Redox System and Its Connection to FAS II and Isoprenoid Biosynthesis

A truely plastid-specific enzyme is the plant-type ferredoxin-$NADP^+$ reductase (FNR) and its redox partner, the iron–sulfur cluster [Fe-S]-containing protein ferredoxin (Fd). This redox couple provides reduced Fd under consumption of NADPH in the apicoplast of *P. falciparum* and *T. gondii* [81] and then serves as an electron donor to acceptor proteins. Currently, the only experimentally confirmed protein receiving electrons from Fd is LytB, the last enzyme of the isoprenoid biosynthesis pathway (Figure 15.2) [82]. However, GcpE, the enzyme acting before LytB, is also a presumed recipient for Fd's electrons [83, 84]. In addition, lipoic acid synthase (LipA) involved in LA biosynthesis is also a likely interaction partner of reduced Fd. This is based on the known reaction mechanism from similar bacterial enzymes of the SAM superfamily [85] and LipA's interaction with Fd in a yeast two-hybrid system (unpublished results). Besides Fd donating electrons to these two pathways (and thus being critically involved in their progression) it is also very likely involved in the apicoplast-resident synthesis of [Fe-S], which also requires an electron-donating system [86, 87]. [Fe-S] synthesis in the apicoplast is presumably an essential process that cannot be replaced by the mitochondrial machinery, and at least five [Fe-S]-containing proteins are know to reside in this organelle, including the mentioned LytB, GcpE, and LipA proteins [88]. Thus, a shutdown of the Fd redox system, either by inhibiting the reductase activity or by preventing Fd from interacting with FNR and/or its acceptor proteins, would be expected to exert a profound effect on more than one essential metabolic pathway. Unfortunately, the only known herbicidal inhibitor class for FNR described to date (substituted benzenesulfonic 3-methyl-2-benzothiazolinylidene hydrazides, e.g., B3; Figure 15.2) is only poorly active against *T. gondii in vitro* ($IC_{50} = 50\,\mu M$) and also not very selective ([88]; unpublished data). The 3D structures of PfFd and PfFNR are known [89, 90] and efforts are under way to search for small molecule inhibitors that would disrupt or prevent Fd from binding to its interacting partners [91, 92]. Given its central role in the described pathways this appears to be a rewarding effort.

New Approaches for the Identification of Novel Antimalarials

The enormous possibilities that high-throughput methods, structural genomics, and proteomic methods offer today for drug target prediction, finding, and validation in general [93–95] continue to have their impact on these topics when it comes to define new chemotherapeutics against malaria. For example, several nonradioactive test systems suitable for high-throughput robotic screens of chemical compound libraries for antimalarial activity were recently described [96, 97]. The initiatives of two structural genomics consortia resulted in a substantial boost of 3D structures available

for *Plasmodium* [98, 99] that can now be probed as drug targets using computational methods. Likewise, proteomic and transcriptomic analyses of the hard-to-study liver stages are coming forward with new ideas about which gene products might present good candidates for intervention [71, 72]. The high-throughput testing of large numbers of approved drugs or protein kinase inhibitors developed for other diseases but now being tested against *Plasmodium* are examples for attempts to streamline the process between drug identification and drug approval [100, 101].

A very exciting development is the possibility to determine and explore the metabolic changes in the host as a result of parasitic infection [102]. Although its immediate use is more on the identification of disease markers and the follow-up of treatment responses [103], one can envisage that metabolomics could also lead to the identification of unique parasite-derived metabolites and their "generators". These might then be exploited as new drug targets. Another interesting group of potential drug targets could be "moonlighting proteins" (i.e., proteins of identical sequence but with different functional roles in a cell [104, 105]). One such example in Apicomplexa is aldolase, which is not only a known glycolytic enzyme but is also an important component of the molecular machinery that allows *T. gondii* and *P. falciparum* to actively invade its mammalian host cell by gliding motility. In this process aldolase acts as a bridge between a cytoskeletal protein (TgMIC2 and PfTRAP, respectively) and actin filaments [106, 107]. Another example of a potential parasite "moonlighting protein" is enolase [108, 109]. The advantage of targeting such proteins is that two or more independent cellular functions could be hit with one compound, increasing the likelihood of observing an effect with lower drug concentrations, provided that the dual activity is targeted by a single compound.

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Part Three
Multicellular Parasites

16
Chemotherapeutic Development Strategies for Schistosomiasis

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Abstract

Schistosomiasis is endemic in 76 countries and territories and infects more than 200 million individuals with almost 800 million people at risk of infection. As a neglected tropical disease, therapy and control relies on just one drug, praziquantel (PZQ). The dependency on a single drug is of considerable concern should development of clinically relevant resistance arise and spread. Accordingly, this chapter summarizes the current state-of-the-art in the development of new antischistosomal small molecules. Attention is also given to molecules that are either in the clinic (e.g., semisynthetic artemisinin derivatives) or under preclinical development (e.g., PZQ analogs, synthetic trioxolanes, alkylaminoalkanethiosulfuric acids). Further, the chapter summarizes efforts to identify lead molecules to validated drug targets (e.g., redox enzymes, cysteine proteases), and finally, the novel application of higher-throughput screening as part of the "hit" identification process.

Schistosomiasis: a Neglected Tropical Disease and the Need for New Drugs

As diseases of the very poor, the neglected tropical diseases (NTDs), such as schistosomiasis, soil-transmitted helminthiases, human African trypanosomiasis (HAT), Chagas' disease, and the leishmaniases, have attracted little pharmaceutical investment despite exerting a huge influence globally on health and welfare [1, 2]. Nonetheless, in the absence of vaccines, the mounting pressures of the toxicity of current therapies and the constant emergence and spread of drug resistance have demanded and produced alternative business strategies to procuring new drugs for some of these NTDs [3, 4]. Thus, the Medicines for Malaria Venture (MMV; www.mmv.org) and the Drugs for Neglected Diseases *initiative* (DND*i*; www.dndi.org),

the latter focusing on HAT and the leishmaniases, exemplify the "public–private partnerships" (PPPs) that have arisen in recent years whereby pharmaceutical industry expertise is combined with government, academic, nongovernmental organization (NGO), and philanthropic investment to support the development of new drugs [5, 6].

For schistosomiasis, however, a dedicated PPP drug discovery and development program does not exist. This is in spite of the disease being one of the most prevalent of the NTDs afflicting over 200 million people in 76 tropical and subtropical countries and territories [7]. The lack of a defined drug discovery effort is due mainly to the significant underestimation of the true burden of schistosomiasis [8] and the availability – since the late 1970s – of a safe and effective oral drug, praziquantel (PZQ). The price of PZQ has plummeted; hence this drug has become affordable and is now recommended by the World Health Organization (WHO) for therapy and control [9]. With this heavy reliance on PZQ comes the obvious concern over the emergence and spread of drug resistance. Even though after 30 years of use strong evidence for PZQ resistance has yet to materialize, parasite lines largely refractory to PZQ have been selected for in rodent hosts [10] and transient parasite tolerance to the drug reported in areas of high transmission in Egypt [11]. Added to this, the Schistosomiasis Control Initiative (SCI; www.schisto.org), working closely with other drug-procurement agencies, is reaching more people more often, thus further elevating the risk of resistance [12, 13]. Nor is PZQ a perfect drug. Cure and egg reduction rates of 100% are rare and the drug acts preferentially against the adult worm, being less effective against the immature schistosomulum stage (plural: schistosomula), thereby necessitating retreatment to remove the burden of those parasites that have since matured [14, 15]. Finally, the WHO has classified schistosomiasis as a category 2 disease, that is, for which new therapies are urgently needed (http://www.who.int/tdr/grants/strategic-emphases/files/matrix.pdf).

Against this background, therefore, it is prudent to search for alternative pharmaceuticals. This chapter reviews some of the key advances in antischistosomal drug discovery and outlines the multifaceted and highly collaborative approach now being undertaken by parasitologists, chemists, bioinformaticians, and screening technologists. We see how key components of the preclinical drug development "pipeline" (validated and "druggable" molecular targets, target-based high-throughput screening, whole-organism (phenotypic) screening, medicinal chemistry and the availability of predictive small animal models) are being put to work to identify drug leads.

What's Old is New Again: Analogs of PZQ

PZQ (**1**, Figure 16.1) was discovered in the mid 1970s by Merck and Bayer [16]. As stated above, the reliance on just one drug to treat schistosomiasis has generated much interest in the elucidation of quantitative structure–activity relationship (QSAR) data for PZQ and the full exploitation of possible analogs.

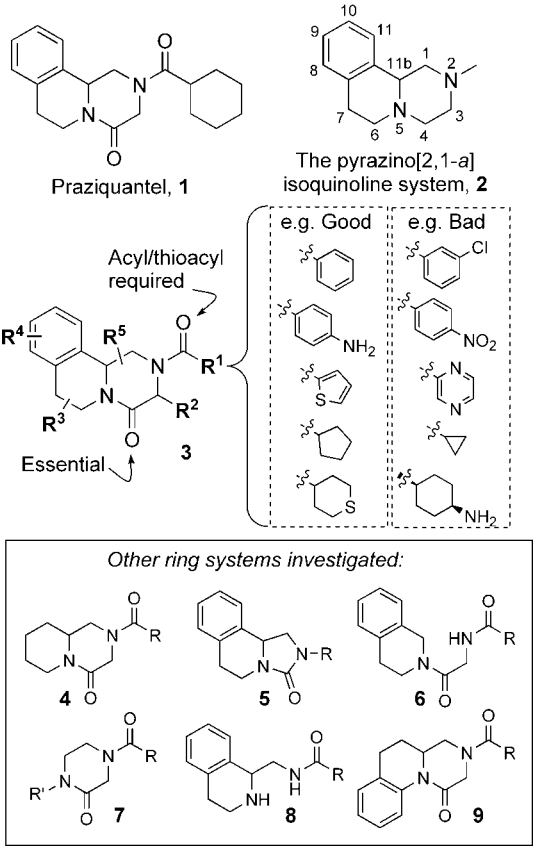


Figure 16.1 Original QSAR for PZQ.

PZQ consists of a functionalized pyrazino[2,1-*a*]isoquinoline ring system (**2**), where an oxo in position 4 is required for activity, as is an acyl or thioacyl attached to position 2 [17]. Beyond this there are five obvious regions for variation (R^1–R^5, **3**). Much of the original medicinal chemistry effort focused on variation in the acyl group of the molecule (R^1). It was found that biological activity was highly sensitive to changes in this region. For example, a benzene ring was tolerated, while 3-chlorobenzene was not; and whereas a cyclohexane ring gave good activity (this molecule being PZQ), other ring sizes gave lower activity. A small number of variations in other parts of the molecule were also tested. Amines in positions 8 and 11 were tolerated, as was a methyl group at position 11b. For the analog in which R^1 = phenyl, methyl groups in positions 6, 7, and 9 decreased activity [17], whereas a methyl at position 3 was tolerated well. A number of other ring systems were also investigated (e.g., **4–9**), none of which gave promising anthelmintic activity. However, many of the data derived from these industrial efforts are not available in the primary literature and must be inferred from patents (variation in R^1: US patent 4 001 411, Merck, 1977, see also German patent DT 2362539, 1975; variation in other positions: US patent 4 051 243, Merck, 1977, see German patent DT 2504250, 1976).

Since the original Merck/Bayer work, there have been few reports of QSAR data concerning other analogs of PZQ. Variation in R^1 was explored with a nicotinyl dipeptide analog of PZQ (**10**, Figure 16.2) that was found to be about two thirds as active as PZQ [18]. Variations in the aromatic ring (R^4) were more recently reported [19]: PZQ with an amine at position 10 (**11**) was weakly active, whereas the corresponding amide derivatives as well as the precursor nitro group at this position gave inactive compounds. It seems clear that R^4 is important for biological activity in addition to R^1. It is still the case, however, that relatively few analogs have been investigated with variation in the aromatic ring. The indole analogs **12** and **13** have been synthesized [20, 21]. Though these compounds were not biologically evaluated in these reports, the original industrial investigations into PZQ analogs suggest that **12**, at least, would be inactive [16]. Variation in R^2 has been largely ignored, which is surprising given that many amino acids are available for incorporation into the structure of PZQ. Todd *et al.* recently synthesized a phenylalanine analog of PZQ (i.e., R^2 = benzyl, **14**), and though this compound showed anthelmintic activity, it was also toxic to mammalian cells, implying a different molecular target (Tamanini, Toubar, El-Fayyoumy, Cioli, and Todd, unpublished results). Positions R^3 and R^5 on PZQ are also largely unexplored.

Regarding the synthesis of PZQ, several methods have been published: the original [16], acid-catalyzed iminium [22] or acyliminium [23–26] ion cyclizations,

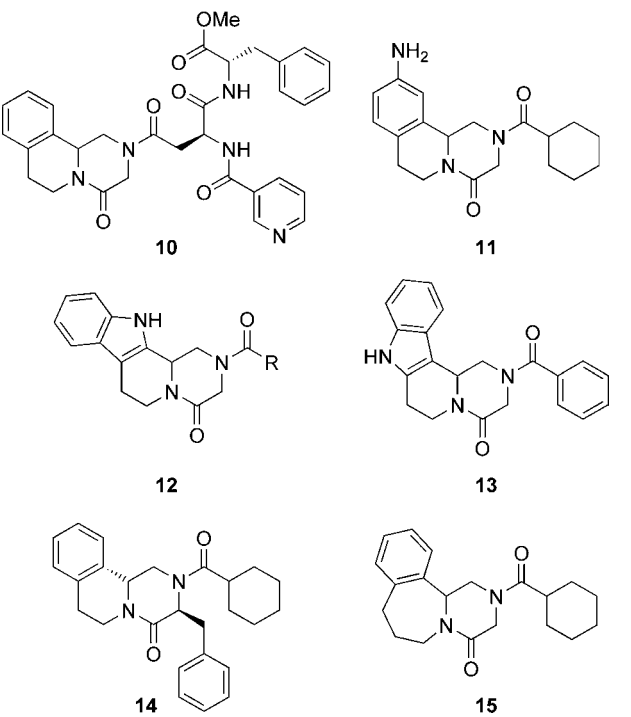


Figure 16.2 Other analogs of PZQ.

an imino Diels–Alder route [27], radical cyclizations [28], and a solid phase approach [29]. Any of these routes could be adapted to the synthesis of PZQ analogs with variation limited by tolerance of alternative substrates to the required reaction conditions. The solid phase synthesis is potentially useful in the rapid generation of large numbers of analogs. The acyliminium ion cyclization method was used in the synthesis of a number of PZQ analogs with variation in R^1 and R^4. These analogs were tested for activity against *Clonorchis sinensis* (a liver fluke) and a similar QSAR was found as for PZQ versus *Schistosoma mansoni* [30]. The seven-membered ring analog **15**, epsiprantel, is used as a cestocide for companion animals [31]. It has also been synthesized using acyliminium ion cyclization [32].

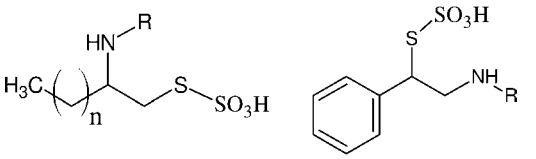
What is perhaps most noteworthy about the overall research into PZQ analogs is that virtually any change in the molecule decreases activity. It is as though the as yet unidentified binding site is a perfect complementary fit to PZQ! A principal component analysis (PCA) of several PZQ analogs came to the same conclusion [33]. With that stated, much of the chemical space around the pharmacophore has yet to be properly explored and/or documented.

All of the above reports on PZQ analogs involve biological activities for racemic compounds. It is known in the case of PZQ that one enantiomer, (R)-(–), is active while the other is inactive [17]. Unfortunately, separation of enantiomers of PZQ is not trivial on a preparative scale; one method in the patent literature involves cleavage of the acyl group prior to a crystallization of a diastereomeric salt and reacylation (German patent DT 2418111, Merck, 1975). Also, PZQ has been synthesized enantioselectively with chiral auxiliary- [34] and chiral catalyst-mediated processes [35]. PZQ may be resolved on a gram scale on microcrystalline cellulose triacetate (MCTA) by applying the methodology described by Ching *et al.* [36] (Intervet Innovation GmbH, personal communication). Synthetic efforts towards enantiopure PZQ will likely also be helpful in the generation of future analogs as single enantiomers, which is almost certainly required for their acceptance by regulatory authorities.

The search for an inexpensive route to the enantiopure material on a large scale is being coordinated by an open source research community known as the Synaptic Leap (http://www.thesynapticleap.org/schisto/projects). The ability to synthesize and administer only the active enantiomer would allow for drug administration at half the current recommended dose regimen (i.e., a single 20 mg/kg oral dose rather than 40 mg/kg) without compromising efficacy and with fewer side effects [37]. A further advantage of a single enantiomer is the possibility of an increase in the standard dose as a means to delay the onset of resistance to PZQ [15] and an improved taste [38].

Alternatives to PZQ – Aminoethanethiosulfuric Acids

During the Vietnam War, the United States army was interested in obtaining drugs for treatment of schistosomiasis. The Walter Reed Army Institute of Research (WRAIR) sent 5000 substances to the research group of José Pellegrino of the



1 2

Figure 16.3 The structures of 2-(alkylamino)-1-alkanethiosulfuric acids (**1**) and 2-(alkylamino)-1-phenyl-1-ethanethiosulfuric acids (**2**) tested for activity against infection by *Schistosoma mansoni*.

Schistosomiasis Research Unit at the Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, Brazil, for screening against *S. mansoni*. Among these compounds was a series of sulfur-containing substances, including 60 aminoalkanethiosulfuric acids (Figure 16.3), that had been developed by the WRAIR as possible radioprotective agents [39, 40]. The screening was performed utilizing the oogram method [41] with the occurrence of a "hepatic shift" of the worms, whereby adult worms, rather than maintaining position in the hepatic portal and mesenteric veins, are shifted into the liver [42]. The oogram method involves the determination of the presence of eggs in all of the stages of development or changes in the proportion of eggs in these stages. Of the 70 acids tested, 10 were active [41]. Only those compounds with a secondary amine and a small or spherical, apolar, aliphatic group attached were bioactive. Also, those derivatives bearing an alkyl or phenyl group alpha to the thiosulfate or amino groups had greater activity than the unsubstituted analogs. Five of the six substances active in mice were also active in hamsters [41]. Based on these results, further variants were synthesized and tested for their antischistosomal activity. Of 10 aminoalkanethiosulfuric acids (**1**) synthesized from propylene oxide, 1,2-epoxybutane and 1,2-epoxyheptane, three were active against infection by *S. mansoni* in preliminary studies.

Antischistosomal Activity of 2-(N-Alkylamino)-1-Octanethiosulfuric Acids (1, n = 5)

In general, the toxicity of these compounds to the host is low [43, 44]. Compounds **1** ($n = 5$) with R = isopropyl and *sec*-butyl were schistosomicidal in the mouse model, inducing an hepatic shift of over 90% of the worms three days after the last day of daily oral treatment with 300 mg/kg for five days (Table 16.1). The hepatic shift occurred between six and 13 days after treatment with these two compounds. During the same period, lesions developed on the worm tegument leading to their eventual elimination [45]. At a single dose of 800 mg/kg, both compounds eliminated over 90% of female worms (mice were sacrificed 20 days after treatment; Table 16.1). Male worm counts were also reduced by 50%.

Oral administration of 500 mg/kg divided into five doses of 100 mg/kg and given at intervals of three hours was as effective as a single 1000 mg/kg dose or five daily doses of 300 mg/kg (Table 16.1). This suggests that the length of exposure to the drug or any active metabolite is more important than the maximum

Table 16.1 Activity of 2-(alkylamino)-1-octanethiosulfuric acids (1, $n = 5$) in mice infected with *S. mansoni*.

Experiment[a] (R)	Drug dose (mg kg^{-1})	Mean ± SD of worms recovered			Worm reduction		Efficacy (%)
		Female	Male	Total	Female (%)	Male (%)	
1a[b] (*sec*-butyl)	1×800	0.4 ± 0.8	2.7 ± 1.7	3.1 ± 2.4	92	45	68
1b[b] (isopropyl)	1×800	0.3 ± 0.5	2.5 ± 1.6	2.8 ± 1.9	93	50	71
1c[c] (*sec*-butyl)	2×800	0.1 ± 0.3	3.1 ± 2.2	3.2 ± 2.5	98	33	65
1d[c] (isopropyl)	2×800	0.0	2.6 ± 2.2	2.6 ± 2.2	100	47	73
Control	—	4.8 ± 1.2	4.9 ± 1.1	9.7 ± 2.3	—	—	—
2a (*sec*-butyl)	1×1000	0.2 ± 0.4	1.9 ± 1.4	2.1 ± 1.6	93	49	70
2b[d] (*sec*-butyl)	5×100	0.3 ± 0.5	1.8 ± 1.5	2.1 ± 1.8	90	51	73
Control	—	3.0 ± 1.0	3.4 ± 2.1	6.4 ± 3.1	—	—	—

[a]The number of the experiment;
[b]groups of 13 mice were sacrificed 20 days after administration of compound;
[c]the two doses were given within a 20-day interval to groups of 13 mice which were sacrificed 20 days later;
[d]the 5 × 100 mg kg^{-1} doses were given at intervals of three hours; both groups of 10 mice were sacrificed 20 days later. $p < 0.05$ when comparing the means of females recovered from group 1a with 1c, and group 1b with 1d. Data from Ref. [48].

plasma concentration. Administration of the drug during the first 10 days after infection had no effect on worm burden, suggesting that immature migrating parasites are refractory to the compounds. Similar results were obtained with the 2-(N-alkylamino)-1-hexanethiosulfuric acids (**1**, $n = 3$), which were synthesized from 1-hexene.

In Vitro Antischistosomal Activity

The aminoalkanethiosulfuric acids (Figure 16.3, **1**, $n = 5$, R = isopropyl and *sec*-butyl) are active against adult worms, schistosomula (immature worms) prepared from cercariae, and schistosomula obtained from the lungs of mice [44]. At concentrations of 0.25 to 1.0 mM, lesions to the tegument and accumulation of hematin in the intestine (the products of blood digestion) are apparent. Hematin accumulation is especially evident in female worms, perhaps due to an inhibition of peristaltic activity. A regression of the vitelline gland is also observed. Upon addition of compound, worms immediately lose the capacity to adhere to the culture dish surface, become paralyzed and die within nine to 12 hours. These compounds also cause paralysis and lesions to the tegument of schistosomula.

After oral administration to mice of radioactively-labeled 2-(*sec*-butylamino)-1-octanethiosulfuric acid (**1**, $n = 5$, R = *sec*-butyl) followed by autoradiography, a variety of metabolites are detected in serum and urine, indicating rapid absorption

and extensive metabolism of the drug [44]. However, the principal metabolite isolated is the corresponding disulfide. *In vitro* studies with adult worms also demonstrate the presence of this metabolite, which is more concentrated in female than male worms.

Studies of Activity Against Strains of *S. mansoni* Resistant to Oxamniquine

Oxamniquine (OXA), a synthetic tetrahydroquinoline, is effective only against *S. mansoni* and was used in national control programs of schistosomiasis, principally in Brazil, resulting in decreased disease morbidity [45]. The emergence of resistance to this compound has been recorded both in field studies and in laboratory animals [46]. Encouragingly, the R1 OXA-resistant strain is not cross-resistant to 2-(*sec*-butylamino)-1-octanethiosulfuric acid. In mice infected with the resistant isolate, this compound (at single doses of 600–800 mg/kg) preferentially removed female worms (90–95%), whereas male worm counts were decreased by 50–60% [47].

Antischistosomal Activity of N-Alkyl-2-Amino-1-Phenyl-1-Ethanethiosulfuric Acids

The above studies pertain mostly to aliphatic compounds. To understand the effect of an aromatic ring on the activity of these compounds, a series of seven 2-amino-1-phenyl-1-ethanethiosulfuric acids [Figure 16.3, **2**, R = propyl (**2a**), isopropyl (**2b**), butyl (**2c**), isobutyl (**2d**), *t*-butyl (**2e**), *sec*-butyl (**2f**), cyclohexyl (**2g**)] was synthesized and tested for activity against *S. mansoni* in female mice infected with 50 cercariae each. Compounds were administered as a single oral dose of 800 mg/kg to groups of 15 mice and animals were sacrificed 20 days after treatment for recovery and counting of worms and eggs [48]. All compounds were active, although a high animal mortality (93%) was observed for **2e**. Like the aminoalkanethiosulfuric acids, the aromatic compounds are preferentially active against female worms (64–100% reduction versus 33–61% reduction in male worms). A 94% reduction in female worms was observed when compound **2c** was administered in a single 800 mg/kg dose to infected animals.

Encapsulation of 2-(Butylamino)-1-Phenyl-1-Ethanethiosulfuric Acid (2d) in a Nanoemulsion

The antischistosomal compound 2-(butylamino)-1-phenyl-1-ethanethiosulfuric acid (**2c**) is poorly soluble in water, which makes its application as a drug difficult. Therefore, a cationic nanoemulsion of **2c** was developed and its *in vitro* bioactivity evaluated [49]. This formulation led to a 16-fold increase in activity against *S. mansoni*. The effect was most likely a result of the interaction between the negative membrane surface of *S. mansoni* and the positive droplets of the nanoemulsion, thus favoring the

penetration of **2c** into the worms. The integrity of the membrane was assessed using the Hoechst 33 258 probe. Female and male worms showed areas of intense fluorescence after their treatment with this delivery system for **2c**. No fluorescence was detected on control worms.

One consistent property of these aminoalkanethiosulfuric acids is the preferred activity against female worms that leads to a reduction or elimination of egg-laying. Overall, their bioactivity is comparable to that of PZQ [17]. Because the disulfide metabolite most probably exerts antiparasitic activity, its encapsulation may represent a way of circumventing toxicity to the host by delivering the compound to the adult worm. Due to their greater activity against female adult worms, in contrast to the preferential efficacy of OXA against male worms [50], a combination of one of these aminoalkanethiosulfuric acids with OXA or PZQ is currently under study.

Alternatives to PZQ: the Artemisinins and Synthetic Trioxolanes

Antischistosomal Activity of the Artemisinins

In 1985, a review was published in *Science* entitled "Qinghaosu (artemisinin): an antimalarial drug from China" [51]. This paper reviewed how Chinese scientists, in the 1970s, had discovered the antimalarial activities of artemisinin, synthesized artemisinin and its derivatives (artemether, artesunate, arteether), assessed their *in vivo* efficacy and toxicity, performed mechanism of action studies, and conducted clinical trials with more than 2000 patients suffering from malaria, including those with *Plasmodium falciparum* strains that were sensitive/resistant to chloroquine. Artemisinin, the active principle of *Artemisia annua*, is a sesquiterpene lactone that contains a peroxide bond in a unique 1,2,4-trioxane heterocycle (Figure 16.4a). The discovery of the antimalarial properties of artemisinin and its derivatives, particularly against multidrug-resistant strains, constitutes the most significant breakthrough in malaria chemotherapy in contemporary times [52]. Indeed, artemisinin-based

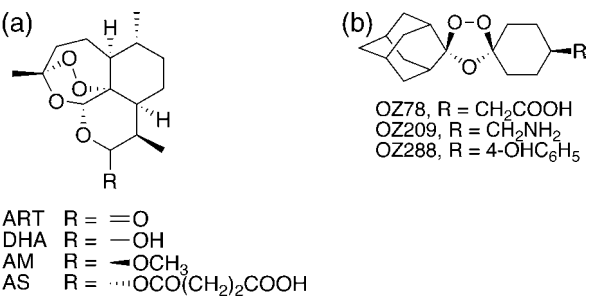


Figure 16.4 Artemisinin (ART) and its derivatives (a; dihydroartemisinin, DHA; artemether, AM; artesunate, AS) and (b) three selected synthetic trioxolanes (OZs).

therapies (ACTs) are now the first line of antimalarial treatments in most malaria-endemic countries: in 2005, over 30 million ACT treatment courses were procured worldwide [53].

Interestingly, in 1980, one decade after the antimalarial activity of artemisinin was discovered, a group of Chinese researchers found that artemisinin also shows *in vivo* activity against *Schistosoma japonicum*. Within a few years, the antischistosomal properties were also confirmed for artemether, artesunate, and arteether. In the late 1980s, *in vitro* and *in vivo* investigations were extended from *S. japonicum* to *S. mansoni*, confirming activity against this schistosome species, and finally, to *S. haematobium*, the third main schistosome species (for a review see Ref. [54]). A number of issues are worth highlighting. First, schistosomula are significantly more susceptible to the artemisinins than adult worms, that is, the opposite stage-specific bioactivity to that described for PZQ. Accordingly, a combination of an artemisinin with PZQ has been suggested in order to target the different schistosome stages in the vertebrate host [55]. Indeed, an artemether-PZQ combination administered to rodents concurrently infected with juvenile and adult *S. mansoni* or *S. japonicum* resulted in higher worm burden reductions that either drug alone [56]. The artemisinins damage the worm tegument, similar to PZQ, but the onset of tegumental alterations is somewhat slower after exposure to artemisinin compared to PZQ [54].

Over the past 10 years, and facilitated by the artemisinins wide use as antimalarial drugs, a number of clinical trials have been conducted with artemether, artesunate, and most recently, ACTs against the three main schistosome species. The key findings of these studies have been reviewed [54, 57]. In brief, the artemisinins at single or multiple doses are safe and in most studies showed a significant effect on patent infections and, most importantly, in preventing the development of new patent infections. There is considerable concern that the use of the artemisinins against schistosomiasis in areas where malaria is coendemic might select for resistant *Plasmodium*. Consequently, the artemisinins are not recommended for the treatment and control of schistosomiasis in such settings. However, the use of the ACTs against malaria is likely to have an ancillary effect on schistosomiasis and this requires rigorous monitoring both at the individual and the population level [54].

Antischistosomal Activity of the Synthetic Trioxolanes

As a drug class, the artemisinins suffer from a number of chemical (e.g., semisynthetic availability, purity, cost) and biopharmaceutical shortcomings (e.g., poor bioavailability, unfavorable pharmacokinetics, such as short half-lives), which limit their therapeutic potential. Synthetic trioxolanes (1,2,4-trioxolanes; OZs; Figure 16.4b) represent a new class of synthetic antimalarial peroxides. Importantly, the OZs have low toxicity, metabolic stability, and good pharmacokinetic properties [58, 59]. Piggybacking from antimalarial to antischistosomal drug discovery revealed that the OZs are also a promising class of novel antischistosomals [60].

Table 16.2 summarizes the efficacies of OZ78, OZ209, and OZ288 against 21-day-old juvenile and 49-day-old adult *S. mansoni* maintained in mice or hamsters.

Table 16.2 Efficacy of selected OZ compounds administered in single oral doses to mice or hamsters infected with juvenile or adult *S. mansoni*.

Compound	Drug administration	Rodent model	Doses (mg/kg)	Worm burden reduction in (%)		Reference
				21-day-old juvenile *S. mansoni*	49-day-old adult *S. mansoni*	
OZ78	Oral	Mouse	Once, 200–400	82.0	0	[60]
		Hamster	Once, 200	80.2	85.0	
OZ209	Oral	Mouse	Once, 200–400	85.0	16.6	[60]
		Hamster	Not done	Not done	Not done	
OZ288	Oral	Mouse	Once, 200–400	95.4	52.2	[60]
		Hamster	Once, 200	86.5	71.7	

Schistosomula were more susceptible to these OZs than adult worms. Single oral 200 mg/kg doses administered to mice infected with 21-day-old *S. mansoni* resulted in total and female worm burden reductions of 82–96%. Whilst moderate total and female worm burden reductions were recorded for OZ288 at a single oral 400 mg/kg dose to mice harboring 49-day-old *S. mansoni* worms (52.2 and 64.9%, respectively), no or low total and female worm burden reductions were found with OZ78 and OZ209 given at the same dose regimen (0–25%) [60]. Interestingly, there is a noteworthy difference in the efficacy of these selected OZs against adult schistosomes depending on the host animal: total and female worm burden reductions following a single 200 mg/kg oral dose of OZ288 and OZ78 were 69.7–71.7 and 85.0–93.2%, respectively, in hamsters infected with 49-day-old *S. mansoni* [60]. Additional research is warranted to further elucidate these observations. The efficacy of OZ78 was also evaluated against 49-day-old adult *S. japonicum* harbored in hamsters and rabbits. A single 200 mg/kg oral dose of OZ78 decreased total and female worm burdens in hamsters by 94.2–100%, whereas a single 15 mg/kg oral dose of OZ78 achieved only moderate total and female worm burden reductions in the rabbit model (40.7–42.3%).

New research is needed to deepen our understanding of the mechanism of action of the artemisinins and the OZs against schistosomes. Preliminary *in vitro* studies with the three main schistosome species suggest a similar mechanism of action of artemisinins to that proposed for *Plasmodium* [61], specifically that iron mediates the cleavage of the peroxide bond, and hence generates free radicals. *In vitro* exposure of schistosomes to a medium containing artemether or OZ78 supplemented with heme resulted in significantly quicker parasite death when compared to a medium without heme [60, 62].

Validated Protein Targets for Possible New Therapies

Thiol Redox Enzymes

Schistosome parasites reside in an aerobic environment and must, therefore, have a means to minimize damage caused by reactive oxygen species produced by their own aerobic respiration as well as by the host immune assault. The schistosome redox system is far simpler than that of its human host. The parasite lacks catalase activity; and analysis of the most recent release of the *S. mansoni* genome sequence (v4.0, 16 May 2007) [63] failed to identify a catalase gene. Defense against oxidative stress in schistosomes relies mainly on the activity of peroxiredoxin (Prx), which is essential to the parasite [64]. Unusually, the activities of schistosome Prx proteins are supported by both glutathione (GSH) and thioredoxin (Trx) [65]. Previous investigations also indicate that both the GSH- and Trx-dependent pathways in *S. mansoni* rely on a single multifunctional flavoenzyme termed thioredoxin glutathione reductase (TGR) that replaces both glutathione reductase (GR) and thioredoxin reductase (TrxR) [66]. No homologs of TrxR or GR are present in the *S. mansoni* genome assembly v4.0.

To validate potential protein targets in schistosomes RNA interference (RNAi) has proven to be a useful reverse genetic approach to silence gene expression [67]. Also, RNAi-based therapies may prove to be a novel approach for the control of schistosomiasis [68]. Targeting TGR by soaking *in vitro*-transformed schistosomula with TGR-specific double-stranded RNA leads to rapid parasite death, either in the presence or absence of oxygen [69]. Silencing Prx expression leads to oxygen-dependent parasite killing; Prx-silenced parasites cultured anaerobically survive equally well as control parasites [64]. However, under increased oxidative stress, Prx-silenced schistosomula die more rapidly. The foregoing studies therefore suggest that the parasite redox pathway is an effective target for the development of new antischistosomal chemotherapies. Two strategies were undertaken by the group of David L. Williams and collaborators to further this hypothesis.

First, Kuntz *et al.* [69] attempted to reposition experimental therapies targeting TrxR, GR (cancer, malaria) and the related enzyme, trypanothione reductase, found in kinetoplastid parasites such as *Trypanosoma brucei* and *Leishmania*. Due to the diverse functions of the TrxR- and GR-dependent pathways, these flavoenzymes are promising targets for drug development to treat these diseases [70, 71]. The knowledge base generated to develop therapies for cancer can be utilized as a "piggy back" starting point to identify novel antiparasitic agents for chronically underfunded NTDs. Thus, small libraries of naphthoquinone and Mannich base inhibitors of TrxR and trypanothione reductase, and known inhibitors of human and plasmodial GR were screened. Among the naphthoquinones, one inhibitor was identified as a selective *S. mansoni* TGR inhibitor, with an IC_{50} of 8 μM, but which was a poor inhibitor of the human TrxR and GR enzymes [69].

The second approach taken by Williams and collaborators was to identify inhibitors of the schistosome redox cascade utilizing resources made available through the NIH Roadmap Molecular Libraries Initiative (MLI; http://www.mli.nih.gov/mli) [72]. The MLI was established in 2004 to address the gap in technologies, expertise,

and cultures between academic and biopharmaceutical organizations by providing academic investigators with pharma-scale infrastructure and technologies necessary to discover starting points for the development, in part, of novel therapeutics. With investigators at the NIH Chemical Genomics Center, a quantitative high-throughput screen of 71 028 compounds comprising the Molecular Libraries Small Molecule Repository and NIH Chemical Genomics Center libraries was completed against the *S. mansoni* redox cascade (Scheme 16.1).

$$\text{NADPH} \rightarrow \text{TGR} \rightarrow \text{GSH} \rightarrow \text{Prx2} \rightarrow H_2O_2$$

Scheme 16.1

The screen, followed by confirmatory and target deconvolution experiments, identified several promising active series, most notably phosphinic amides and oxadiazole 2-oxides, active against the *S. mansoni* antioxidant pathway [73]. The properties of the most active series identified, oxadiazole 2-oxides and phosphinic amides, were investigated in detail [74]. Both chemical series were found to specifically target TGR and not Prx. The activity against *ex vivo S. mansoni* worms of the oxadiazole 2-oxides was linked to the ability of the compound to generate nitric oxide through reaction with TGR. These studies suggest that the mechanism of action of the oxadiazole 2-oxides is based on the production of nitric oxide. The most promising oxadiazole, 4-phenyl-1,2,5-oxadiazole-3-carbonitrile-2-oxide, also known as furoxan (Figure 16.5), was active against all developmental stages (skin, lung, liver, adult) of *S. mansoni*. Furoxan was also active against *ex vivo* adult *S. haematobium* and *S. japonicum*, the other major species infecting humans. Treatment of *S. mansoni*-infected mice with daily intraperitoneal injections 10 mg/kg furoxan for five days resulted in reductions in worm burdens of 99, 88, and 94%, from treatments against skin-, liver-, and adult-stage parasites, respectively. Significant reductions in egg-associated pathologies in the liver and spleen were also seen. The protective effects resulting from treatments with furoxan far exceed benchmark activity criteria set by the WHO for lead compound development for schistosomiasis [5].

The dose–response parameters of the lead furoxan were also investigated. As indicated above, initial studies in *S. mansoni*-infected mice were conducted with furoxan at five doses at 10 mg/kg. Five administrations of lower doses of 1 mg/kg or 5 mg/kg were proportionally less effective, resulting in reductions of 24 ± 6 and $49 \pm 9\%$, respectively, as was a single administration of 10 mg/kg ($13 \pm 5\%$ reduction. However, a single dose at 50 mg/kg was as effective at limiting infections when skin-stage parasites were targeted and provided large reductions ($71 \pm 9\,\%$) when adult-stage parasites were targeted. The higher dose of furoxan is still well tolerated by mice and significantly below the maximum tolerated dose of 250 mg/kg determined for this compound [75]. Collectively, these results suggest that selected derivatives of furoxan could be developed as efficacious, single-dose therapies of

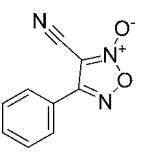


Figure 16.5 The structure of furoxan, compound **9** (NCGC 94 237).

schistosomiasis. Because the mechanism of action of the furoxan is different from that of PZQ, the development of these compounds for use in combination with PZQ may help prevent or delay the onset of resistance to PZQ.

Cysteine Proteases

Papain family cysteine proteases (CPs) have been genetically and biochemically validated as drug targets for a number of parasites, including *Plasmodium* and the African and American trypanosomes [76]. Indeed, for *Plasmodium*, the selection of compounds is well advanced with a number of lead candidates currently in animal models as part of the MMV drug development portfolio. As metazoa, schistosomes express a greater number of possible CP targets, both associated with digestion of host proteins in the gut [77] and elsewhere [78], and it is not entirely clear which, if any, single protease represents *the* target. Nevertheless, the "proof of concept" demonstrations that peptidomimetic inhibitor of CPs are efficacious in animal models of disease [79] maintains interest in targeting this enzyme class. At the University of California San Francisco (UCSF) Sandler Center for Basic Research in Parasitic Diseases, one such CP inhibitor, K777 – well advanced preclinically for treatment of Chagas' disease – removed or significantly decreased *S. mansoni* worm and egg burdens when administered to mice over periods of seven to 14 days [79] with a consequent resolution of liver pathology and function. Using a radiolabeled analog of K777 the main targets were the gut-associated cathepsins B and L. In contrast to the better activity of PZQ toward adult schistosomes, K777 was more effective against the immature migrating schistosomula, thus suggesting that a combination therapy with PZQ is worth investigating.

These data are encouraging, yet the task remains to demonstrate sufficient efficacy when administered over shorter time periods (preferably a single dose) in keeping with the desired target product profile (TPP) for this disease [5]. Within the next year, it is hoped that such tests can be conducted in the baboon model of schistosomiasis and a bulk chemical company has been contracted by the UCSF Sandler Center to produce the necessary K777. Looking beyond K777, a peptidomimetic and non-peptidyl library of approximately 2000 CP inhibitors is now available as part of the UCSF Sandler Center's contribution to drug discovery for treating HAT. These are also available for efficacy testing against schistosomes and will be prosecuted using the newly developed phenotypic screen described below.

Semi-Automated Medium-Throughput Phenotypic Screening

Unlike for many other helminths parasitic in man or animals, the schistosome life cycle can be maintained in small laboratory animals with *S. mansoni* serving as the most widely used model. Thus, the phenotypic evaluation of antischistosomal activity has a long tradition of being performed both *in vitro* and *in vivo* as exemplified in this chapter and cited references. More recently, under the auspices of the UNICEF/

UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR), a Helminth Drug Initiative has been organized specifically to coordinate the testing of compounds against schistosomes and other helminths under more formal conditions [80]. To date, however, compound testing tends to be small-scale given the limitations in worm biomass available and the longer time lines required to generate consistent results compared to protozoa.

In one effort to systematizing schistosome drug discovery, the UCSF Sandler Center has established a medium-throughput, phenotypic screen workflow for *S. mansoni* (Figure 16.6). The screen is designed to integrate smoothly in a 96-well plate format with the compound collections maintained by the contiguous UCSF

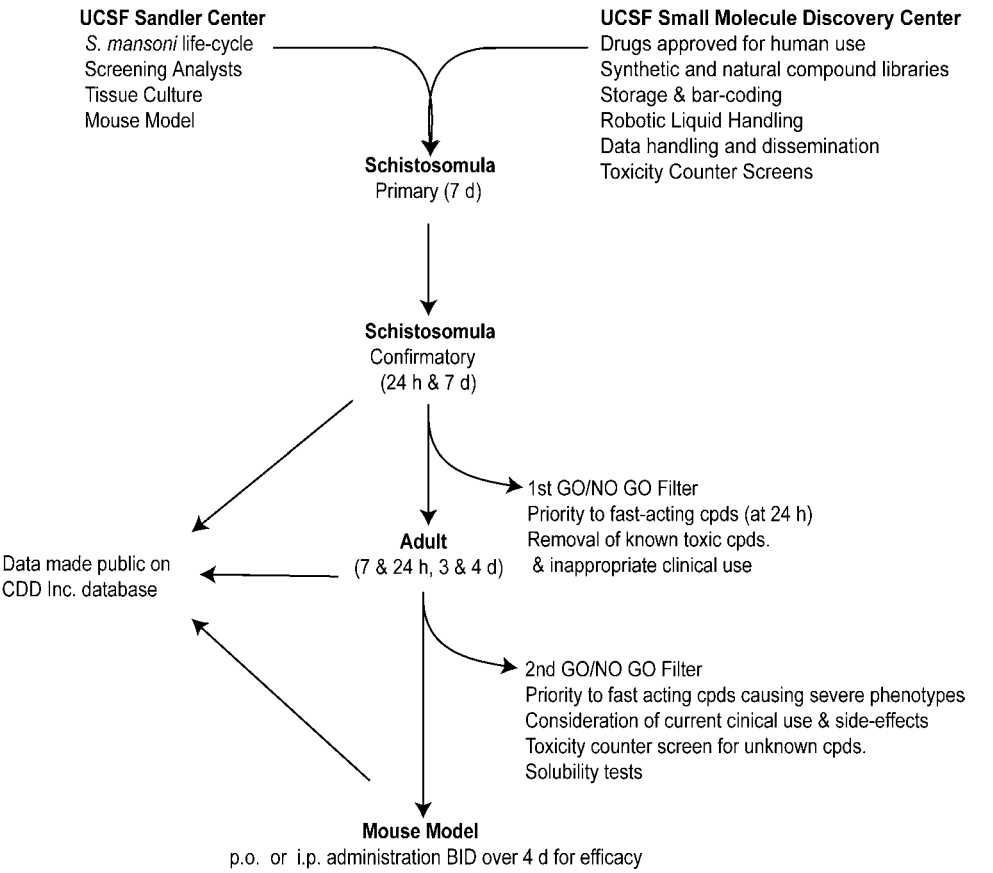


Figure 16.6 Screen workflow employed at the UCSF Sandler Center for Basic Research in Parasitic Diseases screen for identifying antischistosomal compounds. Depending on the size of libraries to be screened, compounds are tested at either one or three different concentrations. Primary screens record phenotypes against schistosomula *in vitro* after seven days. Subsequent "hits" are then put through confirmatory *in vitro* screens in which phenotypes are recorded after 24 hours and seven days. "Hits" arising are then screened against adult worms *in vitro* at varying time-points as indicated. GO/NO GO filters are placed within the workflow to prioritize which compounds go forward, including to efficacy tests in the mouse model of schistosomiasis.

Small Molecule Discovery Center (SMDC; www.smdc.ucsf.edu/). The SMDC houses an HTS facility, searchable small molecules databases, and a medicinal chemistry core. The *S. mansoni* screen workflow takes advantage of the immature schistosomulum stage that can be generated from invasive larvae (cercariae) harvested in their tens of thousands from the intermediate snail host. Phenotypes are assigned visually and "blind" by two analysts working independently. Hit lists are then compared and a "consensus" hit list prepared. After "primary" and "confirmatory" screening against schistosomula, "hit" compounds are then moved forward to tests *in vitro* with adult worms prior to tests of efficacy in a mouse model of disease. GO/NO GO decision filters within the workflow determine whether a given compound goes forward in the workflow by taking into account available clinical data such as toxicity and adsorption, distribution, metabolism, and excretion (ADME). In those cases where smaller numbers of compounds are available, the screen workflow is inclusive of schistosomula and adults, rather than hierarchically prosecuted. Most importantly, all data arising are deposited in a publicly accessible database (Collaborative Drug Discovery Inc.; www.collaborativedrug.com/) that allows for mining and retrieval of quantitative and qualitative data. The phenotypic screen is available as a service to all interested.

Conclusions

The foregoing discussion encapsulates the current dynamism and manifold approaches to identifying lead antischistosomal compounds. Significant progress has been made in the early preclinical drug discovery process: leads have been identified, some of which have defined mechanisms of action, and most display comfortable selectivity indices in vertebrate host models. Thus far, the financial costs involved and time taken have been reasonable by involving extensive piggy-backing of compounds from other industrial and academic discovery programs, including for other tropical parasitic diseases. If these and future leads are to move forward preclinically (e.g., oral bioavailability, pharmacokinetic profiling, safety studies, lead diversification, SAR, employment of other animal models), increased and long-term financial investment, not to mention collaborative scientific oversight are required. Finally, the traditionally high failure rate associated with lead compounds under development as anti-infectives [81] is a constant reminder of the need to maintain and expand the search for new molecular targets and specific small molecules.

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17
Searching New Antiparasitics in Virtual Space

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Abstract

Virtual screening became a credible and complementary tool for HTS during the past two decades. While ligand-based VS approaches were introduced quite early into the drug design process, structure-based methods like high-throughput docking (HTD) could not be used until affordable high-performance computing platforms and 3D structures of the protein targets became available. In this work, we present the single steps involved in HTD by means of two antiparasitic targets – the dopamine receptor CfDopRI and trehalose-6-phosphate synthase. The HTD approach on the dopaminergic receptor was used to generate a target-focused library that was screened afterwards on a HEK cell assay. In the T6PS approach, HTD was used to cross-validate and prioritize the hits that have already been retrieved by *in vitro* screening assays for further examinations in the lead identification process.

Virtual Screening – an Introduction

Computer-based methods, or *in silico* approaches, had already been introduced from the very beginning in rational drug design in the 1980s [1, 2]. Moreover, for a long time the common way of identifying novel, potential lead compounds for further development of drugs was the application of high-throughput screening (HTS). Continuous improvements in HTS technology and its capability to test thousands and even tens of thousands of compounds per day made this technology an essential key component in the drug discovery process for many pharmaceutical companies [3]. However, the establishment and setup of an automated robust target assay using physical compounds typically can generate costs of US$100 000 to US$1 million to produce qualified leads in *in vitro* experiments [4]. Besides, the

random screening of compound collections containing even hundreds of thousands substances, sourced by combinatorial in-house libraries or supplier catalogues, may lead to very low hit rates depending on the target. In addition, supplier collections often contain an undesirably low chemical diversity, and compound ordering can become a very time-consuming process. In-house combinatorial libraries synthesized for HTS also have similar problems, because the chemical space is also very restricted and the whole process is very time-consuming and expensive. Finally, it is simply not possible to test all available, let alone all conceivable (~10 200 structures [4]) compounds, even if there are no limitations regarding time and costs.

To overcome these undesirable limitations novel *in silico* methods, better known by their generic term *virtual screening* [5, 6], have been introduced into the drug design process as credible and complementary methods and alternatives to HTS. In general, virtual screening (VS) can be described as a process of automatic evaluation of large compound libraries in order to prioritize compound subsets by using computer-based applications [7]. Compared to HTS, virtual screening reveals two major advantages: First, by using modern high-performance computing (HPC) platforms, the speed and throughput of *in silico* screenings can be much higher than in experimental setups. Second and more important, VS is not limited to physically existing in-house compound collections. Supplier collections and also synthetically tractable structures from the chemical space of conceivable compounds can be tested without purchase or synthesis, enabling a fast and cheap way to explore unknown parts of the chemical space. The main goal of VS methods is to provide defined activity-enriched datasets that can be assayed by "wet bench" screening setups. This saves time and costs and also diminishes experimental synthesis efforts. In addition, VS can also be used in an already up and running HTS project to offer novel and supplementary insights which allow further prioritization.

Virtual screening involves several computational methods which can be summarized in four classes: 2D property-based approaches, descriptor-based similarity measures, pharmacophore methods, and protein–ligand docking-based approaches.

2D Property-Based Ligand Profiles

Property-based approaches [8] were the first VS methods applied as prefilters in the HTS process. They contain simple counting methods that can quickly calculate properties like molecular weight, number of rotatable bonds or number of H-bond acceptors and donors, but they also include more complex and sophisticated property prediction algorithms. In the beginning, property-based filters, like the popular Lipinski "rule of five" [9], were used to enrich HTS libraries with more drug-like or lead-like compounds. Today pharmaceutical companies also try to predict more complex properties like toxicity, solubility or metabolic stability in early stages of the drug design process to limit the number of failing drug candidates in the more expensive later stages of the development process.

Descriptor-Based Similarity Measures

Searching structurally similar compounds of known active ligands is another strategy for dataset filtering [10]. Similarity measures rest on the assumption that structural similar compounds share similar properties and activities. During this process structures are encoded by descriptors, which can easily be compared by mathematical algorithms. The results of these comparisons are numeric values that describe the level of structural similarity. The ranked results allow the generation of datasets with highly similar structures.

Pharmacophore Approaches

Pharmacophores are defined 3D geometric arrangements of structural fragments or molecular features like hydrogen bond acceptors and donors, charges, aromatic rings or hydrophobic parts, building a spatial key motif relevant for biological activity [11]. In general, this 3D description is obtained by identifying the common features shared by a set of active ligands (ligand-based pharmacophores). Alternatively, pharmacophoric groups can also be extracted from X-ray structures of proteins with bound inhibitors (structure-based pharmacophores). Pharmacophores are not the main focus of this paper, but have been used as additional VS prefilters in this work. Their purpose is mentioned briefly in the corresponding paragraphs below.

Protein–Ligand-Based Methods

Property- and descriptor-based VS methods and also pharmacophore approaches define a ligand profile that can be used as query to filter large compound collections. Therefore, these methods are called ligand-based VS methods. In contrast, protein–ligand-based methods require 3D structural information of the ligands and the protein target during the filtering process and therefore are classified by the term structure-based virtual screening. The most prominent representative of these methods is high-throughput docking (HTD). HTD and its application to identify new antiparasitics is the main focus of this review and are described in detail on the next pages.

High-Throughput Docking

High-throughput docking is based on the method of molecular docking, which tries to predict the optimal orientation of a small-molecule ligand, a “pose”, that is fitted

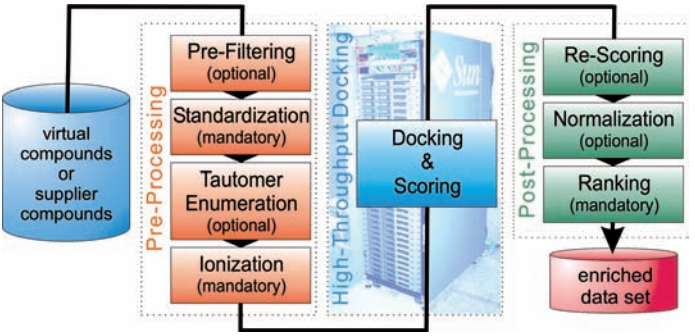

Figure 17.1 Schematic representation of the high-throughput docking process.

into the target binding site to result in an optimum of free binding energy [12]. This process includes all degrees of translational, rotational, and conformational freedom of the ligand and therefore is computationally quite expensive. Nevertheless, docking is only one specific step of many in the process of HTD. In general, the term HTD is used as a synonym for the whole virtual screening process including preprocessing of the ligands and the target structure, docking, scoring, re-scoring, and ranking. Finally HTD comes up with enriched datasets containing potential inhibitors that show a high predicted binding affinity to the target. Figure 17.1 shows an overview of the features involved in this process, which is described in more detail in the subsequent sections below.

Compared to ligand-based approaches, HTD has some additional advantages: First, high-throughput docking is the least biased and most detailed approach to retrieve activity-enriched datasets for a specific target protein. Second, HTD gives detailed insight into the binding mode between ligand and protein and therefore is very helpful to offer novel suggestions on how to move forward in the lead optimization process. Despite these advantages, HTD was introduced rather late into the drug discovery process in the 1990s, because: (i) the required high-quality X-ray structures were not available for many desired targets, and (ii) the massive computing power needed for such approaches did not exist or was not reasonably priced. Today, especially in the human pharmaceutical industry, often one or more 3D structures of the desired target are available. This is caused by the wealth of 3D structural information deposited in public databases like the RCSB Protein Data Bank (PDB; http://www.rcsb.org/pdb/home/home.do) and by the dramatically increased outcome of proprietary and public 3D structure characterization programs like the structural genomics initiative. Furthermore, inexpensive high-performance computing platforms, like Linux clusters, became available recently and are capable of dealing with computationally intensive HTD functions. Beside these advantages, HTD is not always the most suitable VS method to generate a focused dataset [13, 14]. The choice for a specific ligand- or structure-based VS method often depends on several parameters like the quality of data or the specific target protein [15].

HTD in Antiparasitics

In contrast to human target proteins, addressed by pharmaceutical companies, essential parasitic targets are often suboptimally characterized. In the majority of cases, no structural 3D information of these proteins is available. Therefore, virtual screening efforts to identify novel antiparasitics are often restricted to ligand-based *in silico* methods. Despite these limitations, HTD has been successfully applied on some parasitic targets leading to reasonable and valuable results [16–18]. Even if no 3D structure of the target protein is known, HTD can be applied, if high-quality homology models [19] can be generated from X-ray structures of a homologous target protein [18].

In this paper we present the application of HTD on two different antiparasitic targets, trehalose-6-phosphate synthase (T6PS) and dopamin receptor CFDopRI, which are both essential proteins of the cat flea *Ctenocephalides felis* [20, 24]. Both proteins have several limitations regarding structure-based VS methods and therefore are usually not the best choice to explain the different steps of the HTD process. Nevertheless, they represent quite well the standard situation confronting molecular modelers when working on parasitic targets. According to the given knowledge base, the previously collected experience, and the reported structural properties of the targets, two different strategies and goals of the two HTD approaches are described.

The first example, trehalose-6-phosphate synthase, has been shown to be a valid and utilizable target [20–22] to develop novel antiparasitics [23]. The active site of the protein contains two binding pockets, the acceptor pocket and the donor pocket, which have been addressed separately with HTD. In the lack of any existing *C. felis* T6PS X-ray structure, a homology model was generated starting from the 3D structure of T6PS of *Escherichia coli*. Despite the low global sequence identity (37%) between T6PS of *E. coli* and *C. felis*, a suitable 3D model for the HTD approach could be built, because of the high sequence identity of the HTD-relevant binding pockets (acceptor pocket: 100%; donor pocket: 71%). The main goal of the HTD approach was the prioritization and examination of the binding mode of the 193 confirmed HTS hits. By combining the results from HTS, HTD, and an additional pharmacophore model, six hits could be prioritized for ongoing inspection. A detailed description of the involved individual steps is given in the sections below.

The VS approach applied on the second example, the dopamine receptor CfDopRI, can be considered a classic or typical HTD approach. In this case HTD is used as a filter tool to come up with activity-enriched datasets that are starting points for further "wet bench" screening. First, the two dopaminergic receptors (CfDopRI, CfDopRII) of *C. felis* were identified, sequenced, characterized, and validated as appropriate targets for novel antiparasitics [24]. A homology structure of CfDopRII could be established by using the GPCRMod application [25]. Furthermore, a homology model for CfDopRI could easily be derived from the CfDopRII model, because the two receptors are highly conserved, including only one residue replacement within the active site. This 3D structure was used as target protein in the following HTD approach. A database containing 4.2 million purchasable compounds was prefiltered using a pharmacophore profile of a known inhibitor. The resulting focused dataset

contained 7846 compounds and was used in the HTD approach. After performing HTD, 35 structures were identified fulfilling the *in silico* hit criteria. Eighteen of these 35 structures were selected manually to be tested by a human embryonal kidney cell assay (HEK293). Finally, one ligand with high antagonist activity was identified [26].

Defining and Preparing the Virtual Space

Virtual Structures Versus Real Structures

Compounds are three-dimensional and flexible structures that can exist in multiple conformations, tautomer forms, and protonated and stereochemical states. Furthermore, they can occur as neutral single compounds, in different salt forms or aggregated with other small molecular fragments. While these features have no impact on HTS assays, *in silico* screening methods like pharmacophore approaches and especially HTD have to deal with this situation. Correct structural descriptions of ligands are essential to allow a accurate prediction of the binding mode and to discriminate between binders and nonbinders in a HTD approach. In addition we could show recently that the correct structural representation, especially the tautomeric state of structures, might also have a critical impact on pharmacophore-based VS approaches [27]. The preparation of multiple structural *in silico* descriptions is generally addressed in the preprocessing phase of the HTD workflow (Figure 17.1). One of the first steps is the standardization of the ligands, including the removal of counter ions and fragments and the generation of an initial 3D structure. During the next steps multiple tautomeric and ionization states are enumerated. In contrast different conformational and stereochemical states generally do not have to be enumerated in the preprocessing phase, because most applications for HTD and pharmacophore-based virtual screening can flexibly generate realistic ligand conformations and stereochemical states on the fly during the docking process. While the ionization enumeration process can be limited by knowledge-based rules to a small number of reasonable protonation states, limiting the tautomer enumerators to reasonable tautomer forms is difficult. On the one hand, no suitable and fast approaches exist to predict the bioactive tautomer form of a ligand in the active site of a protein. On the other hand, tautomer enumerators considering low energy tautomer forms require computationally very expensive semi-empirical methods. Therefore, they are unsuitable if tens of thousands or hundreds of thousands ligands have to be addressed. In the work presented here, we used a fast, in-house application based on 22 chemical rules that generates an exhaustive number or tautomeric states for each ligand [27].

The impact of the enumeration processes becomes obvious by comparing the numbers of the substances used in the T6PS HTS assay versus their *in silico* counterparts used in HTD. While 130 000 compounds were used in HTS, 468 000 structural descriptions (including additional 240 000 tautomeric and 78 000 ionization states) of

these compounds had to be screened by the HTD approach. While docking one single structure is already computationally expensive, increasing the number of structural descriptions by a factor of 3.6 has to be additionally considered by the high-performance computing platforms used for HTD.

Prefiltering of Datasets

Although screening all purchasable compounds or even all conceivable structures in the chemical space might be a fascinating question, this scenario is not practicable and also not desirable. Because of the extensive computational costs of HTD and the multiple structural representations of each structure to be considered during this process, reasonable prefiltering tools have to be applied in the preprocessing phase to limit the number of ligands. The goal of such filters is to come up with focused, activity-enriched compound collections used for HTD. In general they remove structures from the initial dataset not containing features known to be essential for binding. All ligand-based virtual screening approaches are best suited to solve this task, because they do not require the 3D structure of the target, allow an extensive filtering of compound datasets, and generally are computational efficient and fast. To limit computational costs in the preprocessing phase, prefilters should be used as the initial step in this workflow (Figure 17.1).

Prefiltering of the screening library was not desired in the T6PS example, because HTD was used to cross-validate and prioritize the hits found by high-throughput screening. In contrast in the dopamine receptor example, prefiltering of the database containing 4.2 million compounds was essential to get a reasonably focused library to start with. A pharmacophore query combining substructure elements and molecular shape was generated for this purpose (Figure 17.2d). As a first step in the query generation, the known D1-like selective antagonist SCH23390 was docked into the active site of the CFDopRI homology model (Figure 17.2a) using interaction constraints (Figure 17.2b) derived from the human D2 receptor site chain analysis known in the literature (Figure 17.2c). Afterwards, the docked 3D pose and also the known constraints were used to retrieve the 3D shape features and essential structural features of the inhibitor conformation (Figure 17.2d). By using this query in the program Catalyst ver. 4.11 (Accelrys Inc., San Diego, Calif., USA), 7846 structures could be filtered out from a compound database containing 4.2 million supplier compounds.

Docking and Scoring – The HTD Core Functionalities

After the small-molecule substance collection has been filtered and processed and the protein target structure has been prepared, the essential part of the HTD workflow – the docking application – can be started. However, before screening the complete dataset, several options of the docking application that affect the screening process

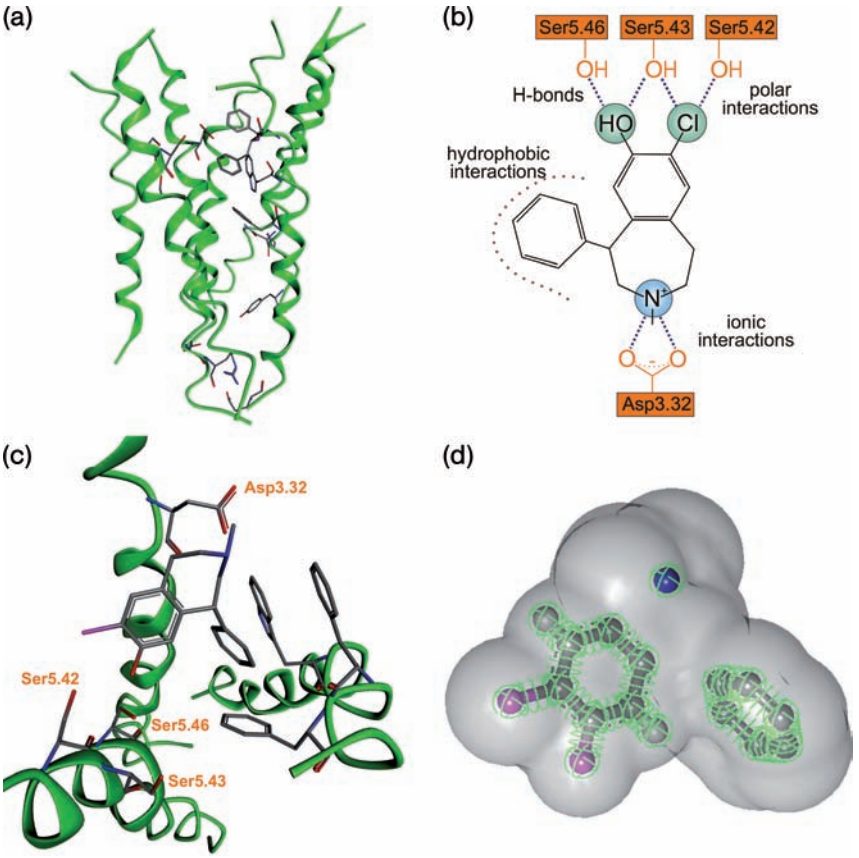

Figure 17.2 Using a homology model of CfDopRI (a) and literature-derived docking constraints (b), the known inhibitor SCH23390 was docked into the receptor structure (c). The resulting target–inhibitor complex was used to derive a specific structure-based pharmacophore model (d) that was used as filter on a database of 4.2 million commercial structures to come up with a focused dataset for the HTD process.

have to be optimized to address the specific characteristics of the protein–ligand interactions. This is basically done by re-docking.

Re- and Cross-Docking – Finding the Optimal Docking/Scoring Parameters

Re-docking or self-docking requires an X-ray structure of a protein–ligand complex. After the ligand has been removed from the active site, the original 3D coordinates

of the isolated ligand are deleted and a new totally different 3D conformation is generated *in silico*. The new conformational representation of the ligand is fitted into the active site of the target protein by the docking application. Finally the results of the docking approach are analyzed regarding its capability to

(a) predict the original bioactive conformation of the ligand within the active site (docking) and
(b) rank this pose as best hit compared to other predicted conformational states (scoring).

By manipulating all docking parameters and analyzing their results in an iterative way, the optimal docking parameters and the best scoring function for the HTD approach can be identified. Moreover, this approach can also be used to validate the quality of a homology model that should be used as target structure for the HTD approach. This specific re-docking case is also called cross-docking [13, 14].

In the T6PS example a homology model has been generated using the X-ray structure (PDB code: 1UQT) of *E. coli* T6PS. This structure also contains an inhibitor, uridine-5′-diphosphate-2-deoxy-2-fluoro-α-D-glucose (U2F), bound into the donor pocket of the protein (Figure 17.3a and c). Docking of U2F was performed on both the original X-ray structure of *E. coli* (re-docking) and the homology model of *C. felis* (cross-docking) using the docking program GOLD [28]. During this process 100 different poses of U2F were docked into the binding pocket, scored, and ranked using nine different scoring functions (GoldScore [28], PLP1 [29], PLP2 [29], LIGSCORE1 [30], LIGSCORE2 [30], LUDI [31], ChemScore [32], JAIN [33], PMF [34]). The difference between original conformation and docked solutions were calculated by using the root mean square deviation (RMSD) method. RMSD measures the differences between the atomic positions of two structures on an atom by atom basis. Re- and cross-docked ligands with an RMSD <0.2 nm are considered as good solutions. For the *E. coli* X-ray structure the PLP2 scoring function [29] performed best, showing an RMSD value of 0.114 nm. Cross-docking the inhibitor into the homology model also led to a quite good solution with an RMSD value of 0.175 nm, indicating that the model is valid and can be used for the HTD approach (Figure 17.3b). Cross-docking has not been used in the GPCR example, because of the lack of an X-ray structure of the receptor–ligand complex.

Docking and Scoring

As already briefly mentioned above, HTD depends on two essential steps: docking and scoring. Docking describes the process of finding poses that contain favorable interactions between the target binding pocket and the ligand. A pose is defined as a specific conformation of the ligand with a specific translational and rotational orientation in the active site. Both, conformation and orientation will be generated during this process automatically by the docking application. Unfortunately, the search space comprises all possible conformations and all possible orientations, which cannot be explored exhaustively. Therefore, docking applications contain

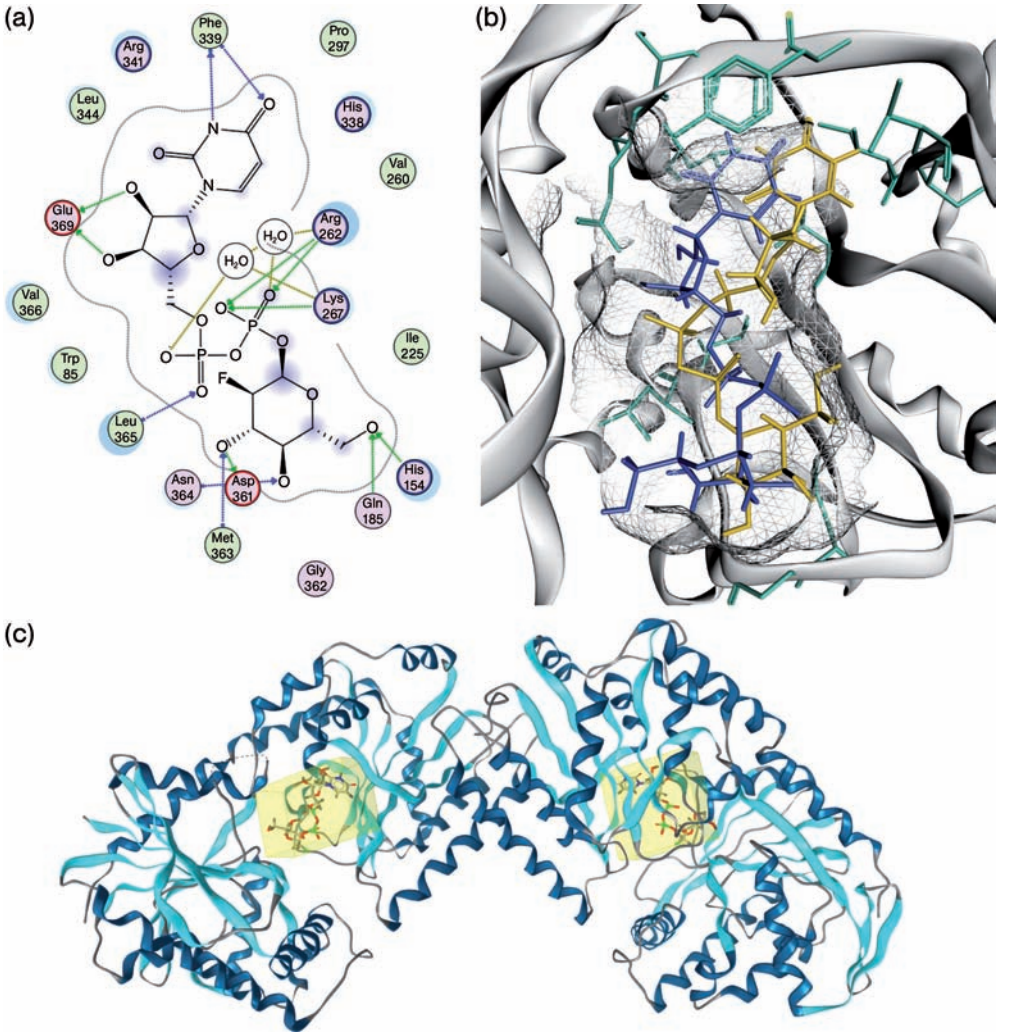

Figure 17.3 Inhibitor uridine-5′-diphosphate-2-deoxy-2-fluoro-α-D-glucose (U2F) bound to the donor binding pocket of trehalose-6-phosphate synthase: (a) T6PS-U2F interaction map, generated by the program MOE ver. 2007.09 (Chemical Computing Group, Montreal, Quebec, Canada), (b) original bioactive conformation (blue) and cross-docked pose (yellow) of U2F in the donor-binding pocket of the T6PS homology model of *C. felis*, (c) U2F inhibitors in the dimer of *E. coli* T6PS.

specific strategies to sample the search space, like genetic algorithms or coarse-grained molecular dynamics simulations [5]. The second and probably more critical feature of docking tools is the scoring function. During the scoring process each single pose is evaluated regarding its binding interactions with the target. As a result a numeric value, representing the extent of the ligand binding affinity, is returned. These numeric representations not only allow presumptions regarding the selectivity

or binding mode of a ligand, but also provide an easy way to rank all poses and ligands screened during the HTD approach. Thus, the accuracy of a scoring function defines the quality of the *in silico* screening approach, because it directly affects the discrimination between active and inactive ligands and therefore the enrichment within the result set. The accuracy of scoring functions has increased in recent years and a broad spectrum of methods for scoring protein–ligand interactions is available. They can be categorized in three main classes: knowledge-based methods, force field-based methods, and empirical scoring functions [12]. The ability of scoring functions to prioritize active ligands varies dramatically and depends strongly on the nature of the target-binding site. As a result no docking program performs comparably well against all target classes [13–15].

As already mentioned above, the docking program GOLD 3.2 [28] has been used for high-throughput screening on T6PS and CfDopRI. GOLD uses a genetic algorithm-based docking method and provides two general empirical scoring functions and an additional kinase-specific scoring function. The HTD job was parallelized using a 64 CPU Linux Compute Cluster (Sun Fire V20z, 2× AMD Opteron 250). In the T6PS example each of the two binding pockets (donor pocket, acceptor pocket) was addressed by a single HTD run using the implemented scoring function GoldScore. In both cases *in silico* screening of the preprocessed 468 000 structural descriptions of the HTS library took 300 hours. In contrast virtual screening of the prefiltered 7846 ligands into the active site of CfDopRI was completed in less than five hours.

Post-Processing – Re-Scoring, Normalization, and Ranking

As already mentioned above, the accuracy of the scoring function has a critical impact on the discrimination capabilities of a HTD approach. However, docking applications basically provide only one or two scoring functions that can be applied to the HTD run. These scoring methods are often not the most suitable functions to evaluate the protein–ligand interactions of the current project. Therefore, it is generally useful to apply various additional scoring functions on all generated poses after the docking run has been finished. This step is called re-scoring, because it is executed after the initial scoring has been performed by the docking application. While in some cases it might be sufficient to identify one suitable scoring function for the whole HTD process by re-scoring, other cases show an improvement of the activity enrichment by combining two different scoring functions: one method to prioritize the best pose for each ligand and another function to accurately separate actives from random compound collections. Another important step during post-processing is normalization. Many scoring functions consider the van der Waals energy between ligands and target protein and therefore automatically favor large ligands with higher molecular weight. To eliminate this effect, it might be useful to normalize scoring results by using specific normalization procedures like taking into account the molecular weight or number of heavy atoms. An exhaustive post-processing approach

ideally considers all kinds of combinations between various scoring functions with and without different normalization methods.

In the re-scoring process of the T6PS example the optimal scoring function should be identifiable by means of its capability to discriminate the confirmed HTS hits from the non actives. Unfortunately, the HTS results did not reveal whether if a hit bound to the acceptor or the donor pocket of the active site of T6PS. However, knowledge about the pocket specificity of the hits was required for the re-scoring process, because HTD specifically addresses each binding pocket. By using a structure-based pharmacophore, derived from the U2F-T6PS complex, a separation of the HTS hits in acceptor pocket-like and donor pocket-like hits could be achieved. These datasets were used to analyze the enrichment capabilities of the various scoring functions. In both cases (acceptor pocket, donor pocket) the best discrimination could be retrieved by using the Ligscore1 scoring function [30] (enrichment of 3.5 within the top 2% HTS hits). By combining the results of the HTS, HTD, and the pharmacophore model, six ligands were selected for ongoing experiments. Figure 17.4a shows the best *in silico* hit (AH135956) in the donor pocket of T6PS exhibiting a similar binding mode as the known inhibitor U2F. The compound was also highly ranked and included in the top 10% HTS hits, and it has been processed in the following steps of the lead identification program.

A totally different approach to discriminate active from non-active ligands is performed by structural interaction fingerprint (SIFt) methods [6]. SIFts are based on a 1D binary string that encodes all essential interactions and known constraints identified in a 3D structure of a protein–ligand complex. This binary representation can be used as a query to identify poses with similar or identical fingerprints.

In the CfDopRI example re-scoring could not be used, because of the lack of known inhibitors. The knowledge about only one inhibitor was not sufficient to identify a suitable scoring function; therefore a SIFt-like approach was used. All *in silico* hits had to fulfill the following constraints: a mandatory interaction with the aspartate residue 3.32 (transmembrane helix: 3, relative position to the most conserved residue in the helix: 32), an optional interaction with one of the two serine residues 5.42 or 5.43, a molecular weight <400 g/mol and a buried surface >85%. Using this filter, 35 *in silico* hits were identified. Eighteen substances were manually selected and tested in a HEK293 cell assay. Finally, one out of the 17 substances showed antagonist activity (Figure 17.4b), which is a great success regarding the suboptimal starting situation.

Conclusion

Virtual screening became a crucial integral part of the drug discovery process during the past two decades. Like HTS, the goal of *in silico* screening methods is to identify novel lead compounds that share a specific pharmacological profile. Although, the two approaches aim at the same goal, they should not be understood as competitors but as credible and complementary tools to each other. The term virtual screening is very handy on the one hand, because it depicts the analogy to HTS. On the other hand

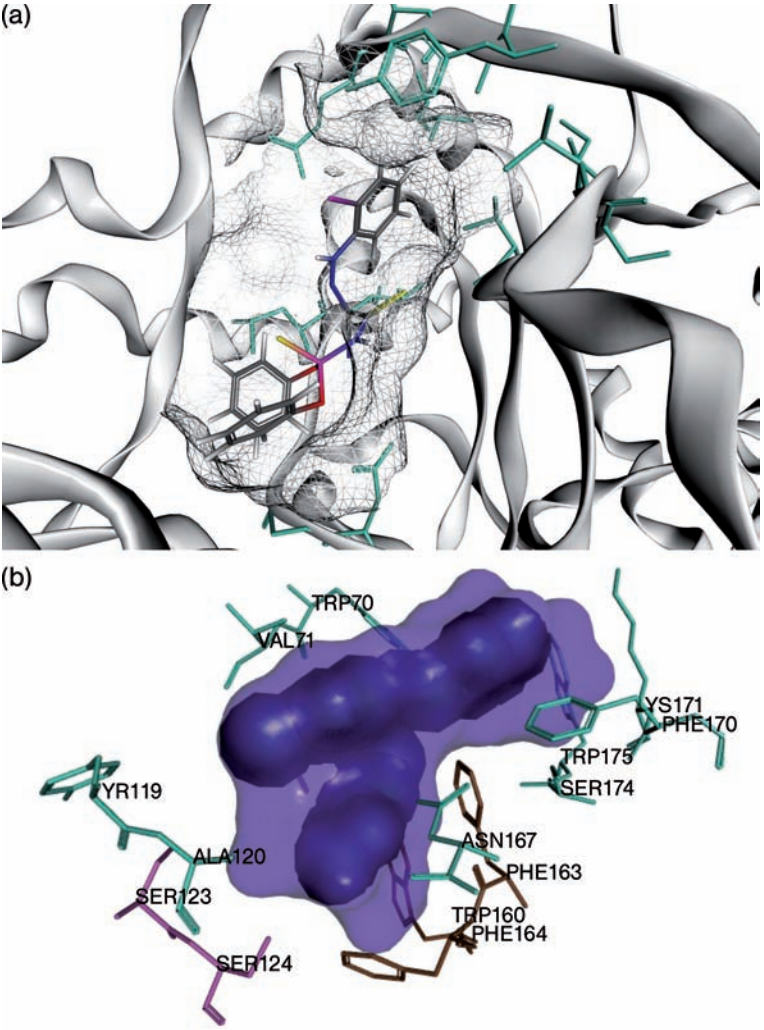

Figure 17.4 (a) Best *in silico* hit AH135956 and known inhibitor U2F in the donor pocket of T6PS. (b) Surface representation (blue solid surface) of the best *in silico* hit docked into the active site of CfDopRI; the semi-transparent surface represents the space covered by all 35 *in silico* hits.

it is somewhat confusing, because it covers a whole bunch of various ligand-based and structure-based *in silico* methods. Some of these methods are described briefly in the first part of this paper. In the second part we focus on one of the structure-based methods – high-throughput docking (HTD) – and its application in the identification of novel parasiticides.

While ligand-based VS approaches were introduced early into the drug discovery process, HTD could not be used until affordable high-performance platforms and

suitable 3D structures of protein targets became available. In contrast to protein targets used in human pharma, the lack of 3D structures is still an issue in the antiparsitics field. Despite these limitations, we present two exemplary success stories where HTD could be used anyhow to identify novel structure classes with antiparasitic activity.

In the first example, VS was used to identify new antagonists of the dopamine receptor I of *C. felis*. Starting from a database containing 4.2 million compounds, 35 potential inhibitors were selected using pharmacophore and HTD approaches. A subset of 17 compounds from this library was selected manually and tested on a HEK cell assay, which resulted in one substance with high antagonist activity. In the second example, HTD was used to cross-validate and prioritize hits that were already identified by *in vitro* screening assays on T6PS of *C. felis*. While the *in vitro* assay could not discriminate between the two potential binding pockets available in the binding site of T6PS, the HTD approach readily identified the binding modes of the potential inhibitors and, therefore, helped to ease the following steps in the lead identification and optimization process. The two examples in our opinion clearly demonstrate the supporting nature of the *in silico* approaches, which to a good part is due to insight on an atomistic level.

Acknowledgement

We thank Sonja Gerber, Simone Lindauer, and Christian Wolf (Intervet Innovation GmbH) for the identification, expression and characterization of the dopamine receptor CfDopRII of *C. felis* and Annette Klinger (Intervet Innovation GmbH) and Didier Rognan (University of Strasbourg, France) for the establishment of the CfDopRI and CfDopRII homology model. We also thank Vincent Madison and Johannes Voigt from Schering-Plough Corp., Kenilworth, New Jersey, USA, for critically reviewing the manuscript.

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18
Cyclooctadepsipeptides – an Anthelmintically Active Class of Compounds Exhibiting a Novel Mode of Action

Achim Harder, Kathryn Bull, Marcus Guest, Lindy Holden-Dye, and Robert Walker*

Abstract

The class of cyclooctadepsipeptides entered the anthelmintic research scene in the early 1990s. PF1022A, the first anthelmintically active member, is a natural compound from the fungus *Mycelia sterilia* which belongs to the microflora of the leaves of *Camellia japonica*. Emodepside is a semisynthetic derivative of PF1022A with a morpholine ring at each of the two D-phenyllactic acids in para position. The main anthelmintic activity is directed against gastrointestinal nematodes of different host animals. PF1022A and emodepside are fully effective against benzimidazole-, levamisole- or ivermectin-resistant nematodes in sheep and cattle. However, the mechanism of action of emodepside appears to be complex, having at least two different targets, a latrophilin-like receptor, and a Ca^{++}-activated K^{+} channel.

Chemotherapeutic Interventions Towards Nematode Infections

Nematode infections are a major cause of human morbidity and losses of disability-adjusted life years (DALYs) in the tropics as well as temperate climates [1]. In animal health, nematode infections especially by trichostrongyle species such as *Haemonchus contortus*, *Trichostrongylus* spp., *Ostertagia* spp., *Cooperia* spp., and so on play a crucial role mainly in cattle and sheep, leading to enormous economic losses [2]. In addition, companion animals are affected by nematodes. Moreover, some nematode infections of companion animals can afflict human as a zoonosis. For example, *Toxocara canis* larvae may lead to toxocariasis in man, which may affect the brain and the eyes [3].

Three classes of broad-spectrum anthelmintics have been available since the early 1960s. These have been repeatedly described in detail by many authors. A recent overview is given in Ref. [4]. In short, thiabendazole was the first benzimidazole introduced into the market in 1963. Since then a wide variety of other benzimidazoles, benzimidazole carbamates, and prebenzimidazoles have been marketed for

the control of nematode infections in ruminants, swine, horses, other food animals, dogs, cats, and humans. Thiabendazole, mebendazole, and albendazole are widely used in human medicine. The second class of anthelmintics are the nicotinic acetylcholine receptor agonists. Members of this class are the imidazothiazole levamisole and the tetrahydropyrimidines pyrantel, morantel, and oxantel. Only levamisole and pyrantel are used in human medicine. Since the mid1970s the new class of the macrocyclic lactones has revolutionized the animal health market [5]. They can be divided into two subclasses: the milbemycins and the avermectins. The common feature of the macrocyclic lactones is their simultaneous activity against nematodes and ectoparasites, leading to the term endectocides. Members of this class are ivermectin, abamectin, eprinomectin, doramectin, milbemycin, moxidectin, and selamectin. Macrocyclic lactones are predominantly used in the livestock industry; however, ivermectin and moxidectin are also recommended for the prevention of dirofilariosis in dogs and cats. Selamectin, the most recent macrocyclic lactone introduced into the market, is registered as a companion animal endectocide for the control of intestinal nematodes, ticks, and fleas in dogs. Until now only ivermectin has been used in human medicine in the control programs for onchocercosis and *Wuchereria bancrofti* filariasis. There are also initial studies using ivermectin for therapy of scabies infections [6]. The situation of anthelmintic treatment in the livestock industry has dramatically worsened through the emergence of resistance against all commonly used anthelmintics. This is reviewed in detail in Chapter 2.

Discovery and Synthesis of PF1022A

In 1992, Sasaki *et al.* reported the isolation and structural determination of PF1022A [7] (Figure 18.1) which belongs to the class of the N-methylated 24-membered cyclooctadepsipeptides, consisting of four alternating residues of N-methyl-L-leucine, two residues of D-lactate, and two residues of D-phenyllactate. This compound (plus its structure, manufacturing, use) was patented by Meiji Seika Kaisha Ltd (Japan) in 1990 (EP 0 382 173 A2). Since August 1990 there have been close contacts between Meiji Seika Kaisha Ltd and Bayer AG (Germany) with respect to research with PF1022A and related compounds. PF1022A is the most active member of related fermentation products isolated from a mycelia cake of the fungus *Mycelia sterilia* PF1022, belonging to the *Agonomycetales*, found in the microflora on the leaf of the plant *Camellia japonica*, collected from the Ibaragi Prefecture in Japan [8]. In 1993 Fujisawa Pharmaceutical Co. Ltd (Japan) filed a patent application which included the bis-para-morphonyl-derivative of PF1022A, which is now called emodepside (formerly PF1022-221; BAY 44-4400; Figure 18.1).

There are very few reports on the total synthesis and structure–activity relationships of cyclooctadepsipeptides [9–13]. There is also a report on the biosynthesis of PF1022A and related derivatives [14]. Meanwhile a variety of derivatives of PF1022A have been synthesized, which are reviewed in Ref. [15]. Interestingly, PF1022A shows

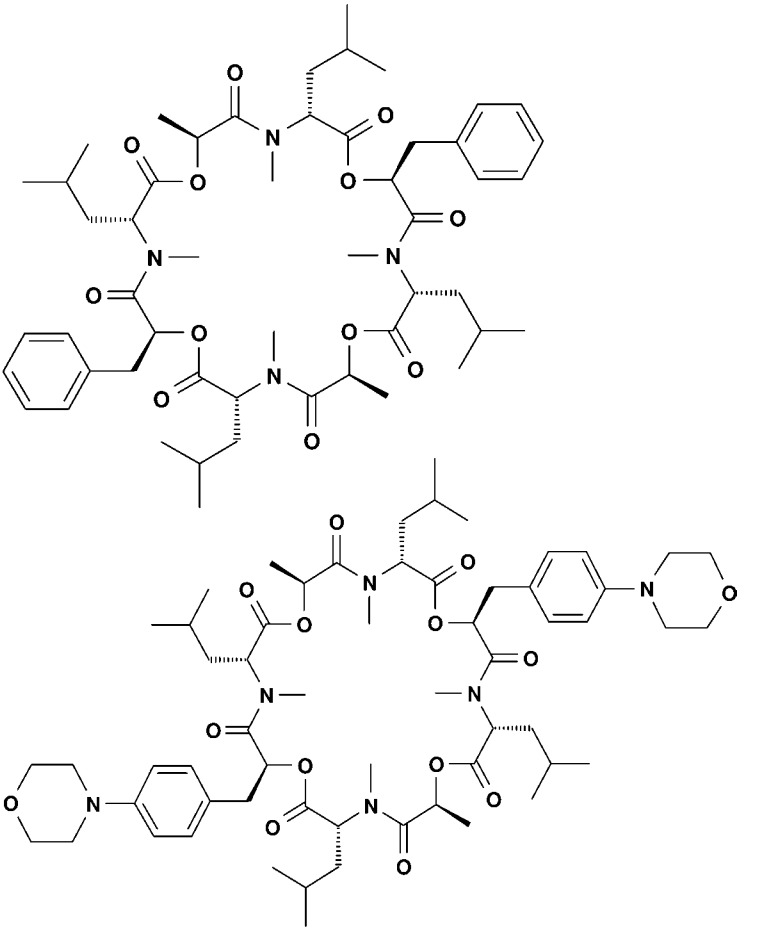

Figure 18.1 Structures of PF1022A (upper) and emodepside (PF1022-221; BAY 44-4400; low).

polymorphism [16]. Four forms of PF1022A, designated as form α, form I, form II, and form III, were prepared. By examining physicochemical properties of these forms by various methods, such as X-ray diffractrometry and differential scanning calorimetry, it became apparent that PF1022A has one amorphous (form α) and three crystalline polymorphic forms. Moreover, in a dissolution study it could be observed that form α and form III had a higher solubility than form I and form II [16].

Anthelmintic Activity of PF1022A

In chicken PF1022A is effective against *Ascaridia galli* at an oral dosage of 2 mg/kg [7, 17]. Against *Toxocara canis* and *T. cati*, intestinal roundworms of dogs and

cats, PF1022A was effective at 0.2 mg/kg given orally [18]. In the gerbil. PF1022A is effective towards *H. contortus*, *T. colubriformis*, and *Ostertagia ostertagi* after oral application (Table 18.1) [19]. In mice, PF1022A is effective after oral application against *Heligmosomoides polygyrus* at 25 mg/kg and against *Heterakis spumosa* at 50 mg/kg [20]. It has no activity towards *Trichinella spiralis* muscle stages or the tapeworm *Hymenolepis nana* at these dosages. In rats PF1022A is fully effective against *Nippostrongylus brasiliensis* at an oral dosage of 50 mg/kg, whereas it is less effective against *Strongyloides ratti*. A good activity is obtained at 100 mg/kg. Moreover, PF1022A shows full anthelmintic efficacy after oral or intramuscular application of 5 mg/kg in dogs experimentally infested with *Ancylostoma caninum*. In horses naturally infested with small strongyles oral treatment with PF1022A at

Table 18.1 Anthelmintic profile of PF1022A and emodepside [19–34].

Host organism	Parasites and treatment used	
	PF1022A	**Emodepside**
Reptiles (snakes, turtles, geckos, lizards, dragon lizards, skinks, sunskinks, varanids)	n.d.	Oxyuridae, Strongylidae, Trichostrongylidae, Ascaridae, Capillaridae
Chicken	*Ascaridia galli*	n.d.
Mice	*Heligmosomoides polygyrus, Heterakis spumosa*	*Heligmosomoides polygyrus, Heterakis spumosa, Trichinella spiralis, Trichuris muris*
Rat	*Nippostrongylus brasiliensis, Strongyloides ratti*	*Nippostrongylus brasiliensis, Strongyloides ratti, Angiostrongylus cantonensis*
Gerbil	*Haemonchus contortus, Trichostrongylus colubriformis, Ostertagia ostertagi*	n.d.
Mastomys	n.d.	*Brugia malayi, Litomosoides sigmodontis, Acanthocheilonema viteae*
Sheep, Cattle	*Haemonchus contortus, Trichostrongylus colubriformi, Cooperia curticei, Dictyocaulus viviparus*	*Haemonchus contortus, Trichostrongylus colubriformis, Teladorsagia circumcincta Cooperia oncophora, Ostertagia ostertagi, Nematodirus* spp, *Trichuris ovis*
Horse	Large strongyles, small strongyles, *Parascaris equorum*	Large strongyles, small strongyles, *Parascaris equorum*
Cat, Dog	*Ancylostoma caninum, Uncinaria stenocephala, Toxocara canis, Trichuris vulpis*	*Ancylostoma* sp., *Uncinaria stenocephala, Toxocara* sp., *Toxascaris leonine, Trichuris vulpis*

5 mg/kg achieves total reductions in faecal egg counts for longer than 5 weeks. Even at 2.5 mg/kg PF1022A, the reduction ranges between 95% and 100% between day 3 until day 35 post-treatment. In sheep PF1022A is highly effective against *H. contortus* at an oral dosage of 5 mg/kg and against *T. colubriformis* at 10 mg/kg. In cattle a full anthelmintic activity can be achieved against the lungworm *Dictyocaulus viviparus* after an oral dosage of 5 mg/kg or after an intravenous dosage of 1 mg/kg. These were the first data indicating that PF1022A has broad-spectrum activity against the major gastrointestinal nematodes of livestock and companion animals [21].

The anthelmintic activity of PF1022A against *Angiostrongylus costaricensis* in the terminal ileum is very much dependent on the route of administration (oral or intraperitoneal), dosage (2.5–40.0 mg/kg) and formulation (solubilized or emulsified) [22]. The anthelmintic activity against this worm is also dependent on the conformation of PF1022A [16]. Thus, the anti-larval effects of the form α or form III is high when given orally and daily for five consecutive days to *A. costaricensis*-infected mice at 10 or 40 mg/kg. By contrast, forms I and II given by the same regimen show little effect against this tissue-dwelling nematode.

Against *Angiostrongylus cantonensis*, residing in the brain of rats and mice, PF1022A does not exert any anthelmintic activity [23, 24]. However, when treatment with PF1022A is performed against adult *A. cantonensis* stages in the pulmonary arteries, a high efficacy can be obtained. Against larvae migrating into the CNS, PF1022A is less effective [25]. It is therefore unlikely that PF1022A passes the blood–brain barrier.

Anthelmintic Activity of Emodepside

In 1997 Fujisawa Pharmaceutical Co. Ltd patented the synthesis of PF1022-221 (emodepside; patent WO 97/02 256). Emodepside is a semisynthetic derivative of PF1022A and exerts a high efficacy against a wide variety of gastrointestinal nematodes in different animal groups, such as reptiles, chicken, rodents, food animals, and companion animals (Table 18.1) [26–34]. This drug is highly effective against the adult stages of the rat nematodes *N. brasiliensis* and *S. ratti*, as well as against the mouse nematode *H. polygyrus*. However, its action against the larval stages of these nematodes varies according to the species. While the drug is highly effective against the lung and intestinal stages of *N. brasiliensis* and *S. ratti*, the larval stages of *H. polygyrus* in the mucosa of the intestine are only partly affected [28]. Moreover, emodepside affects third-stage larvae, fourth-stage larvae or preadult worms of *Acanthocheilonema viteae*, *Brugia malayi*, and *Litomosoides sigmodontis* in infected *Mastomys coucha* when used as a spot-on formulation [35]. Emodepside exerts a species-dependent activity against adult filariae. While *A. viteae* adults show a high sensitivity, repeated relatively high dosages are required to eliminate *L. sigmodontis*, whereas adult *B. malayi* are insensitive.

Emodepside is highly effective against microfilariae of all three filarial species after a single oral, subcutaneous, or spot-on application [36]. Single doses of this drug result in severe pathological changes of intrauterine stages of *L. sigmodontis* or *B. malayi*. These changes may be responsible for long-lasting reductions of microfilaraemia levels, even when curative effects cannot be achieved.

Interestingly, emodepside has been shown to possess resistance-breaking properties against benzimidazole- and levamisole-resistant *H. contortus* strains and against an ivermectin-resistant *Cooperia oncophora* isolate in food animals. Thus, from these experiments, it is conclusive that this drug exerts a completely new mode of action compared to the known anthelmintics.

Emodepside is a component in Profender Spot-on for cats, which has been marketed since the end of 2005. Together with praziquantel it is a product acting against all the relevant nematodes (hook- and roundworms) and cestodes in cats [29–31, 33].

Mechanisms of Action of Cyclooctadepsipeptides

Electrophysiological and Binding Studies

The first studies provided good evidence for a neuropharmacological action of PF1022A, the first reported member of new anthelmintic 24-membered cyclooctadepsipeptides. These studies have been reviewed in detail elsewhere [8, 37–39]. In additional studies PF1022A was shown to interact with GABA receptors of *A. suum* muscle [40]. For direct receptor-binding studies and the elucidation of the possible mode of action a tritium-labeled PF1022A with very high specific radioactivity has been synthesized; and it can now be shown that a receptor, which was unknown at that time, may play a role in the drug's mode of action [41]. This view was supported by the finding that the optical antipode PF1022-001, consisting of N-methyl-D-leucine, L-phenyllactate, and L-lactate, was less effective using *A. suum* membrane preparations than PF1022A.

The Orphan Receptor HC110-R

A target protein for PF1022A was identified using a cDNA library from *H. contortus*. The full-length 3569 bp cDNA, named HC110-R, encodes a protein with 986 amino acids (110 kDa) [42]. Database and phylogenetic analysis revealed 48% identity and 76% similarity of HC110-R with the unknown heptahelical transmembrane 113 kDa protein CEB0457.1 of *C. elegans*. Sequence data and the dendrogram indicate the protein B0457.1 of *C. elegans* is a homolog of HC110-R (Figure 18.2).

Interestingly, HC110-R has about 31% identity with latrophilins from human, cattle, and rat. Latrophilin is a G protein-coupled receptor (GPCR) which was originally isolated from mammalian brain and was also termed CIRL for Ca^{++}-independent

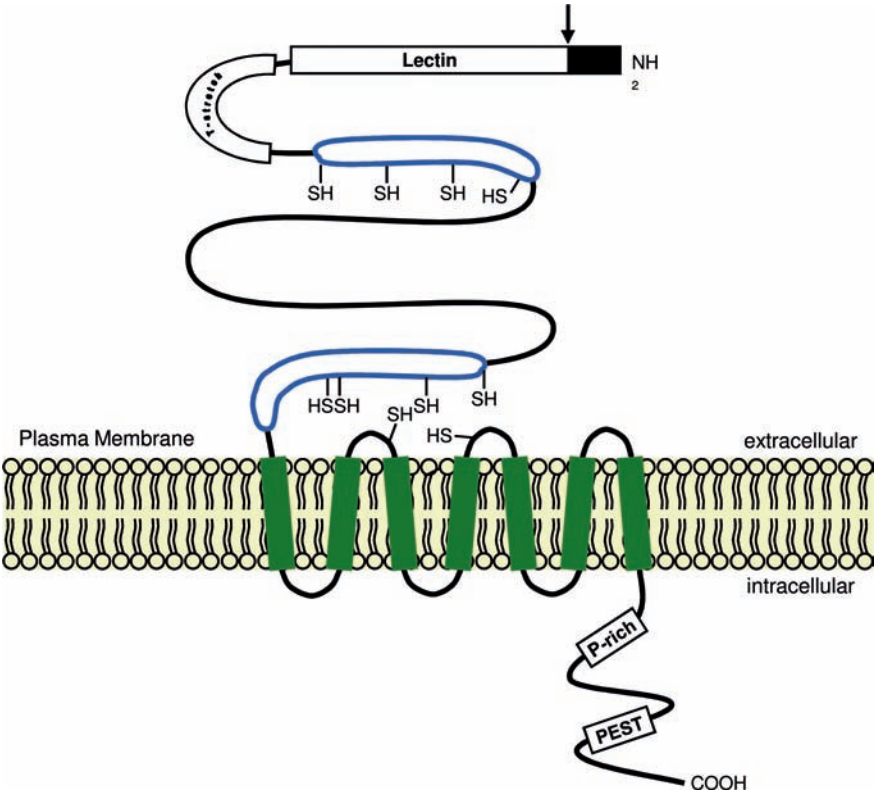

Figure 18.2 Schematic view of HC110-R disposition in plasma membrane.

*r*eceptor of α-*l*atrotoxin [43–45]. Two close homologs, latrophilin-2 and latrophilin-3, were recently identified. The mammalian latrophilins, HC110-R and CEB0457.1, form a highly significant monophyletic subfamily within the secretin receptor family. Further experiments revealed that HC110-R is located mainly in the plasma membrane and also in cytoplasmic acidic vesicles of mammalian cell lines (COS-7 or HEK-293).

Complete coding sequences of putative orthologs were identified in the cattle trichostrongyles *C. oncophora* and *O. ostertagi* [46, 47]. These orthologs were named depsiphilins. The similarity of both depsiphilins to HC110R is 89%, based on the amino acid sequence. The depsiphilins share 46% identity with the latrophilin-like protein 1 (*lat-1*) in *C. elegans* and 47% identity with a hypothetical protein in *Caenorhabditis briggsae*. Recently, a depsiphilin was also identified in the dog hookworm *A. caninum* [47].

α-Latrotoxin Signaling Through HC110-R

α Latrotoxin (α-LTX), the main vertebrate-specific neurotoxic protein of the black widow spider venom, is a ligand of the mammalian latrophilin receptor and it is also a

ligand of HC110-R [43]. In particular, α-LTX binds to the 54 kDa amino-terminal region of HC110-R. Binding of α-LTX to HC110-R induces functional responses by inducing an influx of Ca^{++} which results in increased intracellular free Ca^{++} concentrations. The α-LTX-induced increase of the intracellular free Ca^{++} concentration may be due to influxes of external Ca^{++} and/or release from intracellular Ca^{++} stores. The view is that α-LTX predominantly induced an influx of external Ca^{++}, although there is also some minor release of Ca^{++} from intracellular stores. Moreover, the Ca^{++} influx does not occur through simple diffusion, but rather proceeds through Ca^{++} channels in the plasma membrane. These channels were almost completely inhibited at 10 μM Cd^{++}. In addition, there is a delayed inhibitory effect of nifedipine [42].

Interactions of Emodepside with α-LTX-HC110-R

As for α-LTX, binding of PF1022A to HC110-R also occurs at the N-terminal 54 kDa region of the receptor [42]. Binding of emodepside to HC110-R also influenced α-LTX signaling through HC110-R. The Ca^{++} influx induced by α-LTX was diminished and delayed in the presence of emodepside. This effect could not be observed in the presence of the optical antipode of PF1022A – called PF1022-001 – which is anthelmintically ineffective. Specificity of these findings is further supported by the fact that emodepside did not affect the Ca^{++} response of nontransfected HEK-293 cells to other neuropharmacologically active drugs such as carbachol, isoproterenol, or arecoline mediated through the endogenous natural M1-R, β_2-R, or nicotinic receptors in HEK-293 cells, respectively.

Emodepside Action on Pharyngeal Pumping in *Caenorhabditis elegans*

With HC110R transfection in HEK cells, only the interactions of emodepside with the receptor could be investigated. There is no possibility to examine the downstream signal transduction cascade inside the cell. Further experiments to reveal the mechanism of action of the cyclooctadepsipeptides PF1022A and emodepside were therefore conducted using *C. elegans* as a model. This model is suitable because HC110R not only possesses 48% identity and 76% similarity with the heptahelical transmembrane 113 kDa protein B0457.1 (*lat-1*) of *C. elegans*, but furthermore *lat-1* of *C. elegans* is the homolog to HC110-R. We also know, by using embryonated *C. elegans* eggs and preimmunsera directed against the N- or C-terminal regions of HC110-R, that the homolog *lat-1* protein is located in the pharynx of *C. elegans* [42]. Therefore, it would be interesting to know whether emodepside impairs the physiology of pharynx pumping. Indeed, emodepside causes an inhibition of pharyngeal pumping in *C. elegans* in a concentration-dependent way, with an IC_{50} value of 6 nM (Figure 18.3). Thus, emodepside might exert its mode of action via *lat-1*, the latrophilin-like

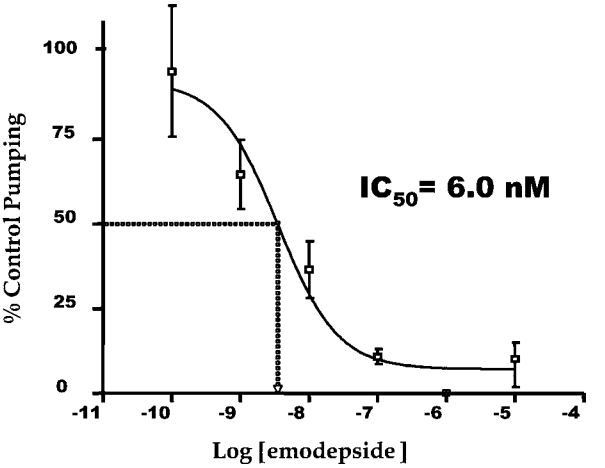

Figure 18.3 The effect of emodepside on *C. elegans* pharyngeal pumping.Emodepside causes a concentration-dependent inhibition of pharyngeal pumping with an IC_{50} of 6 nM.

receptor located in the pharynx of nematodes. The importance of an intact pharynx physiology for the viability of nematodes has been shown in different papers, summarized by Prichard 2001 [48]. Here, ivermectin, which activates various glutamate-gated chloride channels including AVR-15 (GluClα2), acts directly through paralysis of the pharynx, although this is not sufficient for the anthelmintic action of ivermectin. Thus, a similar situation can be expected in the presence of emodepside and related cyclooctadepsipeptides.

Emodepside Acts in *Caenorhabditis elegans* by Activation of α Latrophilin Receptor

To investigate the role of the presynaptic signal transduction cascade in emodepside's action, a variety of mutants for presynaptic proteins were determined [49, 50]. Among these mutants were *egl-30*(*ad806*) with a loss-of-function in the Gαq protein. Pharyngeal pumping as well as locomotion were less sensitive to the drug in these mutants compared with wild-type controls. This crucial experiment indicates that the latrophilin receptor couples to Gαq in mediating the effect of emodepside in *C. elegans*.

Different mutants were selected to test their sensitivity to emodepside, for example, *egl-8*(*md1971*), *egl-8*(*n488*), and *goa-1*(*n1134*). Phospholipase C-β is encoded by *egl-8*, while *goa-1*(*n1134*) is a Gαo loss-of-function mutant. Both *egl-8* mutants were less sensitive to emodepside on pharyngeal pumping and locomotion. By contrast, the *goa-1*(*n1134*) mutants were more sensitive to emodepside on both functions.

These results strongly support the view that emodepside activates a latrophilin receptor which acts through a phospholipase C-β pathway to hydrolyze phosphati-

dylinositol-4,5-bisphosphate (PIP_2) to produce diacylglycerol (DAG). This process is negatively regulated through the effect of Gαo. DAG acts on UNC-13 to increase the amount of readily releasable pool of neurotransmitter. The prediction is that this neurotransmitter relaxes pharyngeal as well as body wall muscle, resulting in flaccid paralysis of the nematode [50]. From experiments with *A. suum*, it is suggested that emodepside preferentially releases a neuropeptide [38, 39].

Effects of the Novel Anthelmintic Emodepside on the Locomotion, Egg-Laying Behavior, and Development of *C. elegans*

The previous research has clearly demonstrated that latrophilin-like receptors are involved in the effect of emodepside on pharyngeal muscles. In these earlier studies it could be shown that emodepside affects locomotion of the L4 larval stages of *C. elegans*, as well as the rate of the worm's development and the adult worm's egg-laying behavior. In a series of additional experiments these effects were quantified [51].

Indeed, locomotor behavior of wild-type *C. elegans* L4 larval stages is less sensitive than that of adults to emodepside. The decreased sensitivity of L4 larval stages to emodepside is seen with both body form and generation on agar (adult IC_{50} 3.7 nM; L4 IC_{50} 13.4 nM) and thrashing behavior in liquid (adults 16%; L4 worms 48%). Continuous exposure of wild-type *C. elegans* to emodepside slows gross morphological worm development. This effect is emodepside concentration-dependent. The rate of worm hatching from eggs on agar plates containing emodepside was not significantly different to controls. This suggests that it is development post-hatching rather than hatching itself that is affected by the drug.

Emodepside also appears to suppress wild-type *C. elegans* egg-laying behavior, with acute exposure to the drug at 500 nM resulting in an almost total inhibition of egg laying within the first hour. The number of eggs generated by worms each hour when exposed to emodepside was not significantly different to unexposed *C. elegans*, resulting in the former becoming bloated with eggs and eventually rupturing. This suggests that the effect of emodepside on reproduction is not due to an inhibition of egg production but is rather a paralytic effect on the egg-laying muscles. These results, when combined with previous results, suggest that the neuromuscular junction is the major site for the action of emodepside on the body wall muscles, pharynx, and egg-laying muscles [51].

Action of Emodepside via SLO-1 Channels in *Caenorhabditis elegans*

As latrophilin null animals are not resistant to the effects of emodepside on locomotion, it is clear that emodepside does not act solely through a latrophilin-

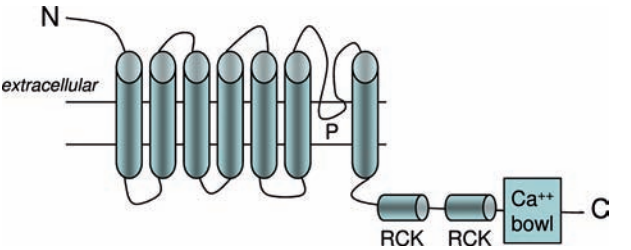

Figure 18.4 Structural cartoon of a SLO-1 channel subunit. SLO-1 consists of seven transmembrane domains, a membrane domain P which directly contributes to pore formation, two intracellular regulatory domains RCK and a C terminal calcium ion binding region, the Ca^{++} bowl. Each channel consists of four of these subunits which assemble in the membrane as a tetramer to form a central potassium selective pore. For the position of the mutations conferring resistance to emodepside see Ref. [54]).

dependent signaling pathway [50]. To determine further effectors for emodepside, forward genetic studies were conducted that employed EMS chemical mutagenesis to generate highly emodepside-resistant strains [52]. The genetic loci conferring resistance were identified using SNIP-SNP mapping techniques. This mapping technique, and subsequent sequencing, indicated that resistance to emodepside was conveyed in nine strains by either deletions or point mutations in the Ca^{++}-activated K^{+}channel gene, *slo-1*. A reference allele for *slo-1, js379*, which is a putative functional null mutant for the channel SLO-1 (Figure 18.4) [53] was also shown to exhibit high-level resistance to emodepside [54]. This indicates that the inhibitory effect of emodepside on *C. elegans* behavior is likely to involve activation of a SLO-1-dependent pathway. The most parsimonious model, which remains to be unequivocally established, is that SLO-1 is an emodepside receptor. Thus, emodepside appears to have a complex mode of anthelmintic action, involving a presynaptic latrophilin-dependent pathway and a necessity for the SLO-1 channel.

SLO-1 as Target for Emodepside

Large-conductance calcium and voltage-activated potassium channels, termed SLO-1, have an important role in the regulation of neuronal and muscle cell excitability in both vertebrates and invertebrate animals. Their physiological role, structure, function, and pharmacology have thus been the subject of extensive investigation [55]. It is intriguing that emodepside acts to activate these channels and it is pertinent to consider how this might impact on signaling at the neuromuscular junction which results in inhibition of behaviors which are important for nematode survival.

In the nematode *C. elegans* functional null mutants for *slo-1* are viable, indicating this gene is not essential for survival. However, their behavior differs from wild-type animals, consistent with the proposal that SLO-1 regulates the excitatory output of neural networks, particularly locomotion [reviewed in Ref. [54]]. The genetic analysis of emodepside's mode of action indicates that excessive signaling through SLO-1 is

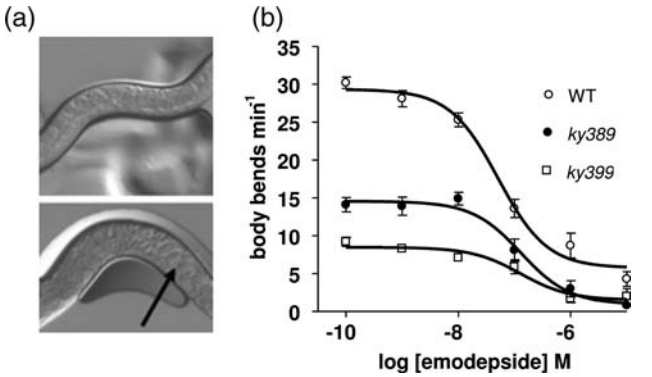

Figure 18.5 Gain-of-function of slo-1 mutants. It is predicted that the mutants ky389 and ky399 have increased calcium sensitivity and increased channel activity, respectively (reviewed in Ref. [55]). (a) The top panel shows the gonadal region of an adult hermaphrodite wild-type worm and the lower panel shows the same region in slo-1(ky389). Note the wider girth of the mutant which is due to egg retention (indicated by the arrow) and that this is similar to the effect of emodepside on wild-type animals [55]. (b) A graph showing the locomotor activity of adult wild-type and slo-1 hermaphrodites animals, off food, in the presence of increasing concentrations of emodepside. Note that both mutants move at a slower basal rate than wild-type animals; however both are inhibited by emodepside in a similar fashion.

the cause of the pleiotropic actions of this anthelmintic which results in profound inhibition of pharyngeal, body wall, and egg-laying muscles. This is supported by analysis of *C. elegans slo-1* gain-of-function mutants which exhibit a similar, though less severe, phenotype to emodepside-treated animals (Figure 18.5).

Towards an Integrated Model for the Anthelmintic Action of Emodepside

This model is based on the premise that *C. elegans* is an adequate and informative model for the mode of anthelmintic action in parasitic nematodes. Furthermore, a model to explain the mode of action of emodepside should account for the inhibitory effects of the drug on locomotion, feeding, egg-laying, and also for the observation that it slows development.

It is clear that high-level resistance to emodepside is observed in animals that are absent or deficient in signaling for the calcium-activated K^+channel, SLO-1. These animals appear to be resistant to all the pleiotropic effects of emodepside and suggest that SLO-1 is directly involved in the drug's action, perhaps by acting as a receptor. Although SLO-1 is required for the inhibitory effect of emodepside on both locomotion and feeding behavior, there is evidence of a difference in the precise action of emodepside at the body wall and the pharyngeal neuromuscular junction which regulates these behaviors. Thus, at the body wall neuromuscular junction emodepside can signal through SLO-1 either in motorneurones or in muscle to inhibit locomotory behavior. However, at the pharyngeal neuromuscular junction, SLO-1 expressed in the

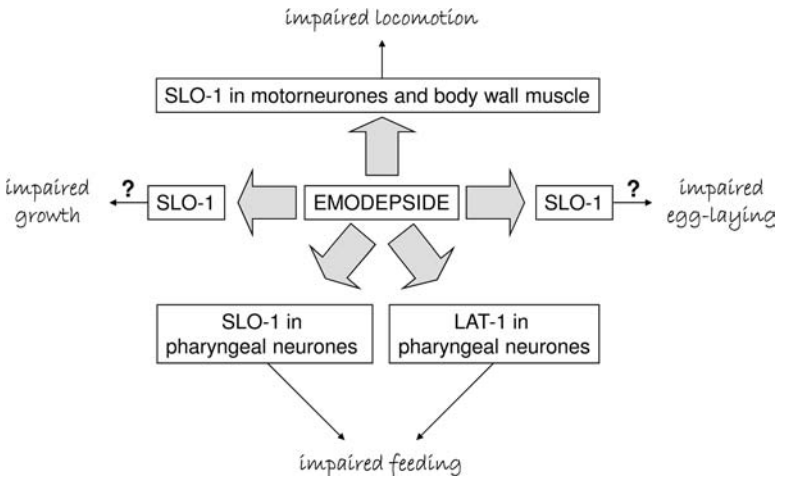

Figure 18.6 Effects of emodepside and the role of SLO-1 and LAT-1 as molecular effectors. SLO-1 null mutant animals are highly resistant to the inhibitory effects of emodepside on growth and development, feeding, locomotion, and egg-laying. Therefore, SLO-1 is required for the pleiotropic anthelmintic actions of emodepside. The effects on locomotion can be mediated by SLO-1 present either in the body wall muscle or the neurones however, the effects on feeding are mediated by neuronally expressed SLO-1 [54, 55]. Expression cloning experiments have also implicated the G protein-coupled receptor, latrophilin in the mode of action of emodepside [42, 50]. lat-1 null mutants show reduced sensitivity to the effect of emodepside on the pharynx and therefore latrophilin may contribute to the inhibitory action of emodepside on feeding. The precise role of SLO-1 in mediating the effects of emodepside on growth and egg-laying have not yet been defined. It is possible that the effect on growth may be secondary to reduced feeding and poor nutrition.

muscle is not sufficient to confer sensitivity to emodepside and here the effect is mediated by SLO-1 expressed in neurones [54, 55].

In addition to this, there is evidence that emodepside can bind to another putative effector molecule, latrophilin [42]. Although a *C. elegans* functional null mutant for latrophilin (*lat-1, lat-2*) remains sensitive to the inhibitory effects of emodepside on locomotion, it is less sensitive to the effect of emodepside on the pharynx. This suggests that emodepside causes inhibition of feeding via a neuronal SLO-1-dependent pathway which is facilitated by *lat-1* (Figure 18.6).

Conclusion

Compounds of the new class of the cyclic octadepsipeptides are broad-spectrum anthelmintics. The action of the parent compound PF1022A as well as the semisynthetic derivative emodepside is directed against a wide variety of nematodes in various target animals. PF1022A exerts activities against gastrointestinal nematodes

and lungworms. Emodepside has additional activities against different extraintestinal filariae.

Our current understanding of the mode of action of emodepside is that it acts to impair neuromuscular transmission in nematodes. This results in an inhibition of vital behaviors, namely feeding, locomotion, and egg-laying. It does this predominantly by activation of a calcium-activated potassium channel signaling pathway and indeed the evidence, although indirect, suggests that this channel, SLO-1, is a receptor for emodepside. An additional emodepside receptor, the latrophilin HC110-R, has been expression cloned from *H. contortus*. Whilst this receptor seems less important for the direct inhibitory action of emodepside on neuromuscular transmission, there is evidence that an interaction of emodepside with this receptor could contribute to its anthelmintic effect.

Analysis of *C. elegans* mutants for proteins involved in regulating neuromuscular release suggest that the action of emodepside signaling through the latrophilin pathway involves a phospholipase C-β and hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) to produce diacylglycerol (DAG). DAG thereafter acts on UNC-13 to increase the amount of readily releasable pool of neurotransmitter and this neurotransmitter, possibly a neuropeptide, inhibits the activity of the pharyngeal muscle.

Overall, the evidence unequivocally supports a key role for SLO-1 in the inhibitory effect of emodepside on locomotion and feeding. However, it appears that the precise action of the drug at the body wall and pharyngeal junction are different. At the body wall neuromuscular junction, the drug can signal through SLO-1 either in motorneurones or in the muscle to inhibit locomotion. By contrast, at the pharyngeal neuromuscular junction, muscular SLO-1 is not sufficient for emodepside's action. Here the effect is mediated by SLO-1 expressed in neurons. Furthermore, it seems that the inhibitory effect of emodepside on feeding of the nematodes occurs via a neuronal SLO-1 dependent pathway which is facilitated additionally by the latrophilin, LAT-1.

Important questions still remain with respect to the molecular interaction of emodepside with SLO-1 and the role of these channels in parasitic nematodes of veterinary importance and the putative network between SLO-1 and *lat-1*. Nonetheless, it is clear that SLO-1 and latrophilin are new targets for anthelmintic resistance-breaking treatment of nematode parasites.

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19
Chemical Optimization of Anthelmintic Compounds – A Case Study

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Abstract

Anthelmintic resistance has become a major threat for the treatment of diseases in the veterinary field, particularly in farm animals. In the past three decades, only one anthelmintic compound class addressing this resistance problem has reached the market, and new chemical treatment options are urgently needed. This report describes a chemical optimization approach of a screening hit that led to the discovery of a novel class of anthelmintically active compounds. In addition to retaining the target site inhibitory potency of the newly synthesized compounds, physicochemical property prediction tools were used during the synthesis planning phase in an attempt to overcome permeation difficulties. By combining these two strategies, the nematocidal activity of the investigated class of compounds was significantly improved.

Lead Structure Identification

Known Anthelmintic Drug Targets

Parasitic nematodes are a major cause of disease in livestock and in companion animals, and they lead to considerable morbidity and even mortality with concomitant distress and economic losses. Effective prophylactic or therapeutic vaccines are virtually nonexistent. Thus, prevention and treatment of nematode diseases rely largely on chemotherapy with anthelmintically active compounds [1]. The molecular modes of action known or thought to be responsible for the therapeutic effects of antinematode drugs include agonistic action on nematode ion channels, such as nicotinic acetylcholine receptors or 4-aminobutyric acid (GABA)- and glutamate-gated chloride channels, inhibition of parasite acetylcholinesterase, blockade of nematode microtubule polymerization, uncoupling of mitochondrial proton gradients, and interaction with latrophilin-like G protein-coupled receptors [2–5] (see also Chapter 6; Table 19.1).

Table 19.1 Antinematode compound classes and their known or hypothesized *in vivo* targets [1–7, 14].

Compound class	Representatives	Nematode *in vivo* target
Tetrahydropyrimidines	Pyrantel, morantel, oxantel	Agonists of nicotinic acetylcholine receptors
Imidazothiazoles	Levamisole, butamisole	Agonists of nicotinic acetylcholine receptors
Benzimidazoles	Fenbendazole, tiabendazole, albendazole	Inhibition of microtubule polymerization
Macrocyclic lactones	Ivermectin, selamectin, moxidectin	Agonists of glutamate-gated Cl^- channels
Organophosphates	Coumaphos, naphtalophos, dichlorvos	Inhibition of acetylcholinesterase
Cyclooctadepsipeptides	Emodepside	Interaction with latrophilin-like receptors, interaction with Ca^{2+}-activated K^+ channels
Piperazine	Piperazine	Agonists of GABA-gated Cl^- channels
Salicylanilides	Closantel, rafoxanide	Uncoupling of mitochondrial proton gradients
Chlorinated sulfonamides	Clorsulon	Uncoupling of mitochondrial proton gradients

The use of modern anthelmintics has resulted in efficient control of virtually all economically important veterinary nematodes over the past 40 years. Resistance, however, continues to emerge in key trichostrongylid parasite species like *Haemonchus contortus* or *Teladorsagia circumcincta* of sheep as well as in cyathostomes and *Parascaris equorum* of horses. This resistance has developed against both single and multiple compound classes, such as benzimidazoles, imidazothiazoles, or macrocyclic lactones and causes great concern [6–12]. Veterinary parasitologists in practise have consequently expressed an urgent need for new resistance-breaking chemical classes in the anthelmintic drug arsenal to counter the threat of untreatable nematode infections [13] (see also Chapter 2).

The only novel resistance-breaking anthelmintic compound class recently introduced in the companion animal market are the cyclooctadepsipeptides (Table 19.1) [5] that most likely act on latrophilin-like receptors of nematodes, although in a recent report Ca^{2+}-activated K^+ channels have been proposed as alternative targets [14] (see also Chapter 18). Their therapeutic and commercial potential in livestock species, which are even more susceptible to nematode resistance, is still unclear. Other compounds reported to be effective against resistant nematode infections are the paraherquamides, as well as other structurally related marcfortines [15, 16]. These compounds are thought to block nematode nicotinic acetylcholine receptors. A third class of compounds useful against resistant nematode infections includes inhibitors of the respiratory chain (reviewed in Refs. [17–21]). The discovery, isolation, and characterization of the fungal metabolite nafuredin at the Kitasato Institute in 2001 provided the first convincing *in vivo* validation of a nematode

respiratory chain component as an effective anthelmintic drug target [22]. It was shown by Omura and co-workers that nafuredin possesses potent and nematode-selective inhibitory activity against complex I of the respiratory chain and that it is efficacious in a sheep model of *H. contortus* infection. Unfortunately, the structure of nafuredin and its complex total synthesis [23] is a major obstacle for chemical optimization programs.

In this article, we report the discovery of a novel class of compounds having inhibitory activity against nematode complex I with *in vitro* antiparasitic efficacy. Compared to nafuredin, these compounds are substantially simpler to synthesize. We describe the optimization of the lead compound by a combination of chemical synthesis, chemoinformatic tools, and biochemical and biological screens.

Identification of Screening Hits

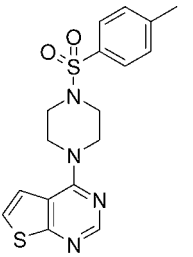
Hit compounds can be obtained from different sources, with medium-throughput functional screens offering the big advantage of selecting hit compounds with substantiated pharmacokinetics, thus potentially accelerating any later lead optimization program. To determine anthelmintic activity *in vitro*, we established a test systems using readily accessible larval stage 3 (L3) of the gut-dwelling parasitic nematode *Ascaridia galli* which is the intestinal roundworm of chicken. In this screen, the anthelmintic effects were visually classified by assessing mortality, morphological damage, decreasing motility, slowing progression of development, and neutral red uptake by larvae. All information collected were combined to a comprehensive phenotypic assessment that may, as the case arises, also permit a preliminary mode of action assignment. The potency of compounds was recorded as the minimum effective concentration (MEC), with a compound being considered a hit structure when activity was detected at a test concentration of 25 μM or less.

From this screening campaign, compounds **1–3** (Figure 19.1) emerged as promising candidates. Intriguingly, these compounds show close structural similarity as they all have a thienopyrimidine heterocyclic core, an aliphatic spacer moiety, and a terminal aromatic group in common. All initially found hit compounds were remarkably active in the *A. galli* larval test system, exhibiting inhibitory effects in the range of 0.19–25 μM. Most remarkably, compound **1** exhibited activity comparable to the marketed anthelmintic agent Levamisole (MEC = 0.78 μM).

Based on visual structural comparisons with known respiratory chain inhibitors, such as pesticides fenazaquin or pyrimidifen [24], we hypothesized that the mode of action of the screening hits could be related to the interruption of the mitochondrial respiratory chain at the complex I site.

The Respiratory Chain as a Promising Anthelmintic Drug Target

Energy metabolism is essential for the survival, growth, and reproduction of all living organisms, including parasites. The aerobic respiratory chain is the key



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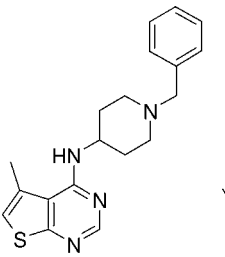

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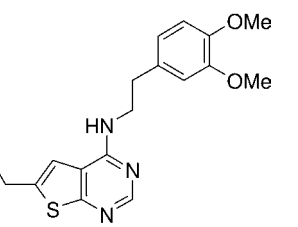

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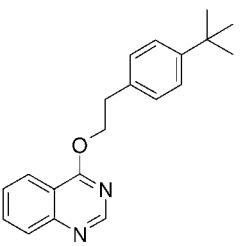
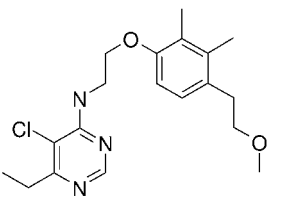
MEC (*A. galli* L3)	0.19 μM	3.12 μM	25 μM



Fenazaquin



Pyrimidifen

Figure 19.1 Hit compounds and structurally related inhibitors of complex I.

energy-transducing mechanism. It functions as a pathway mediating outward electrogenic translocation of protons through bacterial or mitochondrial membranes, resulting in a proton gradient across the membrane [25]. The proton motive force generated by the respiratory chain drives ATP synthesis by F_0F_1-ATPase. The basic mechanism of this process is essentially unchanged from bacteria to human mitochondria. Some parasites, however, have developed variants of energy-transducing pathways to adapt to the environmental conditions within their natural hosts. Typically, the mitochondrial respiratory systems of parasites are more variable than the corresponding pathways of host animals [21, 26, 27].

The size of the parasitic helminth is important in determining the type of energy metabolism used. An inverse correlation between body size and the aerobic capacity of nematodes has been observed, most likely due to limitations of glucose and oxygen diffusion into parasite tissues. Small nematodes residing close to the relatively oxygen-rich gut mucosa, such as *Nippostrongylus braziliensis*, possess a functional aerobic respiratory chain. Those nematodes use oxygen as a terminal electron acceptor [28]. In contrast, larger nematodes dwelling in the oxygen poor lumen of the gut, such as *Ascaris suum*, tend to have alternative electron transport chains and electron acceptors [21, 26].

The respiratory chain of mitochondria from free-living nematode larvae has been observed to be comparable to that of the mammalian host. Reducing equivalents from respiratory chain substrates, such as NADH and/or succinate produced by the citric

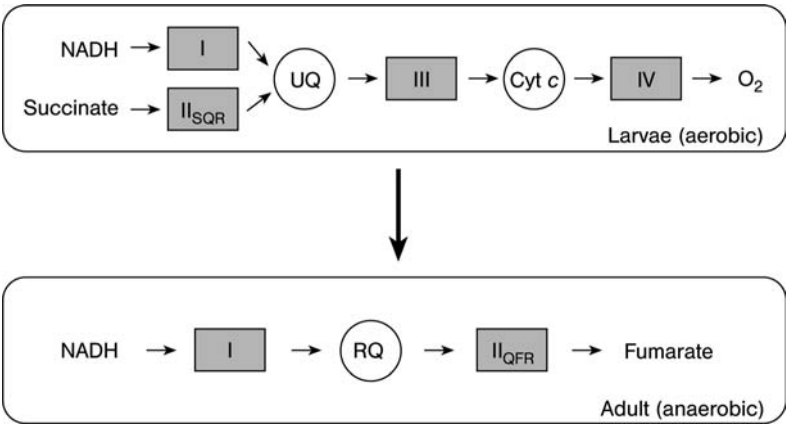


Figure 19.2 Change of the respiratory chain during the life cycle of *A. suum*.I, complex I (NADH-UQ oxidoreductase); II_{SQR}, complex II (succinate-UQ oxidoreductase; SQR); III, complex III (ubiquinol-cytochrome c oxidoreductase); IV, complex IV (cytochrome c oxidase); UQ, ubiquinone; RQ, rhodoquinone; II_{QFR}, complex II (quinol-fumarate oxidoreductase; QFR; modified from Kita *et al.* [21].)

acid cycle are transferred to ubiquinone (UQ) *via* the inner mitochondrial membrane dehydrogenase complexes I (NADH-UQ oxidoreductase) and II (succinate-UQ oxidoreductase, SQR; Figure 19.2) [29]. The reducing equivalents are then transferred from UQH_2 to UQH_2-cyctochrome c oxidoreductase (complex III), and *via* cytochrome c, to cytochrome c oxidase (complex IV). The cytochrome c oxidase oxidizes reduced cytochrome c, using oxygen as a terminal electron acceptor, to form H_2O.

Figure 19.2 also shows the mitochondrial respiratory chain of adults from some nematode species. It is immediately obvious that their respiratory chain is strikingly different from that of the corresponding free-living larval stages. The reducing equivalents of NADH are transferred *via* the complex I to rhodoquinone (RQ), which possesses a much lower standard reduction potential compared to the canonical UQ. The reducing equivalents are transferred from RQ to a modified complex II, which acts as quinol–fumarate oxidoreductase (QFR) (Figure 19.2) [29]. Finally, the electrons are transferred by the modified complex II to fumarate [30], which acts as terminal electron acceptor and is reduced to succinate. This reverse reaction direction of complex II requires a different set of iron–sulfur clusters incorporated in complex II [29, 31]. Furthermore, the low potential RQ (midpoint potential $= -63$ mV) is used as an electron carrier from complex I to complex II instead of the high potential UQ (midpoint potential $= +110$ mV) of the aerobic electron transport chain of the larvae [32]. The energy efficiency of the anaerobic mitochondrial electron transport pathway is much lower than that of the aerobic electron transport pathway. However, it provides adult nematodes from some species, such as *A. suum*, with more energy than lactic acid formation or ethanol production, which are the common mechanisms for NADH reoxidation under microaerophilic or anaerobic conditions.

The electron transport chain provides a number of possible interaction sites for nematocides. Use of complex I as a molecular target, as in this study, has the advantage that both adult and larval stages of the parasitic nematodes are targeted. A potential drawback of complex I inhibitors, however, is a probable toxic effect on the electron transport chain of the parasite's vertebrate host. For this study, the NADH-fumarate oxidoreductase (complex I/modified complex II; ETC I/II) activity from adult *A. galli* was chosen as the nematode molecular screening target, due to the accessibility of worms, as well as the robustness and straightforward readout of the assay. Application of this assay allows for the identification of inhibitors of both complex I (expected to act on both aerobic and anaerobic nematode stages) and modified complex II (expected to be specific for anaerobic nematode stages). Using this test system, half maximal inhibitory concentrations (IC_{50}) of hit compounds **1–3** on ETC I/II of *A. galli* were determined and found to be 2.4, 33, and 166 nM, respectively. This coincides with their relative *A. galli* L3 *in vitro* potencies (Figure 19.1).

Lead Structure Optimization

First Optimization Cycle – Initial Structure–Activity Relationships

In order to identify and to determine the chemical groups responsible for evoking the ETC specific biological effect in the nematode larvae, the preliminary structure–activity relationship (SAR) of the newly identified thienopyrimidines was compiled. For this purpose, the most potent hit compound (compound **1**) was selected as the lead structure, and an initial library was designed for further testing. The compound structure was divided into three regions (Figure 19.3) with all regions being addressed sequentially, varying only one motif at a time: first the aromatic head group (defining region 1), followed by the central diamine linker (defining region 2), and

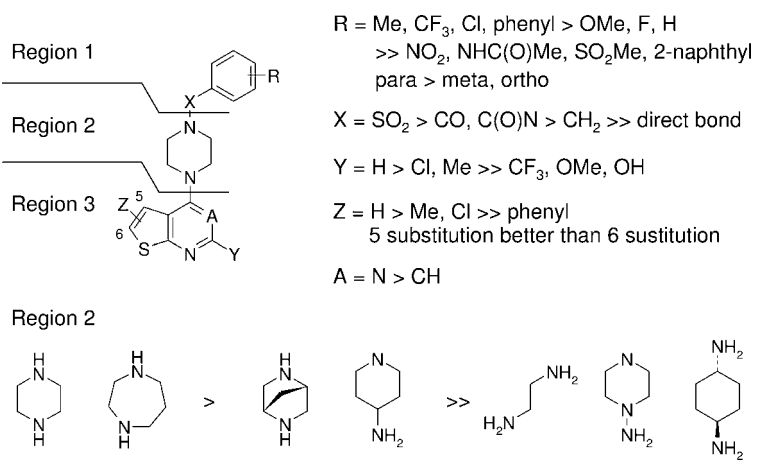


Figure 19.3 Structure–activity relationship (SAR) of thienopyrimidine analoges.

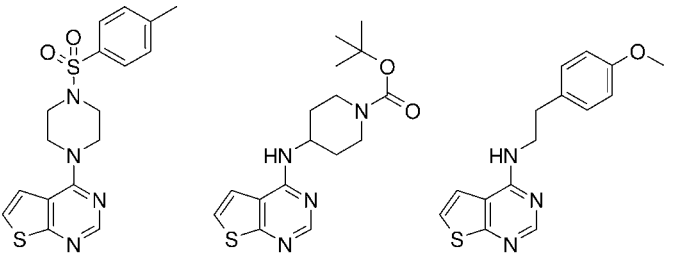
finally the thienopyrimidine core (defining region 3). The anthelmintic potencies were determined by application of the *A. galli* L3 *in vitro* assay and also of an analogous test comprising larval stages 3 and 4 (L4) of *Oesophagostomum dentatum*, the nodular worm of swine. To facilitate comparison of an increasing flow of activity data, a combined "bioscreen score" based on the MECs of all larval species was deduced, ranging from 0 to 18. By applying this scoring method, the marketed anthelmintic agents levamisole and ivermectin, that were used as standards in this study, exhibited scores of 13 and 18, respectively.

Following this structural fragmentation (Figure 19.3), a library of approximately 200 compounds was assembled using readily accessible 4-chlorothienopyrimidines as starting materials. Figure 19.3 summarizes the nematocidal SARs of compound **1** analoges. For region 1, substitution of the para position on the aromatic motif led to analoges with superior activity, and of those substituents, methyl, trifluoromethyl, chloride, and phenyl were observed to be best. In addition, derivatives consisting of sulfonamides ($X = SO_2$) were more potent than the corresponding amides, ureas, or methylene-containing derivatives. In cases where no spacing group X was employed, a significant decrease in activity was observed. Compared to the other regions, alteration of region 2 had the greatest though negative impact on activity. While activity was only retained with homopiperazine, all other variations yielded reduced potencies. During this stage of research, no other linking entities carrying less than 2 nitrogen atoms were investigated. In region 3, alteration of the heterocyclic moiety was not well tolerated and proved to be deleterious for nematocidal activity. Even the introduction of small substituents, such as methyl or chloride, for Y or Z resulted in compounds with significantly lower activity, while the use of other substituents, such as phenyl or hydroxyl, resulted in generally inactive compounds. Changing the orientation of the thiophene ring of the heterocyclic ring system also reduced activity, and a pyrimidine moiety ($A = N$) was by far superior to the corresponding pyridine core ($A = CH$).

Due to the lack of improvement of on-target and/or nematocidal activity in this first round of derivatization, the optimization approach was revised and a new set of compounds was synthesized in which two regions were varied simultaneously. When tested, compounds carrying any region 3 modifications showed no improvement of nematocidal activity. However, variation of the spacing moiety (region 2) in conjunction with another group in region 1 yielded compounds with increased potencies. Two illustrative examples, aminopiperidine **4** and phenylethylamine **5**, are presented in Figure 19.4. Although both compounds show significantly higher nematocidal activity than lead compound **1**, their inhibitory potencies towards the target complex were similar, suggesting that these derivatives possess superior pharmacokinetic properties. As both compounds belong to proprietary structural classes that are patented as pesticidal entities [33, 34], they were not considered as starting points for the lead optimization process.

Improvement of Larvicidal Activity by Optimization of Physicochemical Properties

In order to be active against nematode larvae *in vitro*, a potential drug must be able to interact with a specific molecular target structure crucial for worm viability or



	1	4	5
IC_{50} (ETC I/II *A. galli*)	2.4 nM	3.2 nM	2.7 nM
MEC (*A. galli* L3)	0.19 µM	0.006 µM	0.04 µM
Bioscreen Score	12	16	14

Figure 19.4 Derivatives of compound 1 with improved nematocidal activity.

motility, and it must be able to achieve sustainable and effective concentrations at the site of action. Thus, optimal drug transfer into the parasite must be considered early on when designing drugs to combat nematode infections. Because it is presumed that passive diffusion through the external cuticular is the common mechanism of drug entrance into nematodes, lipophilicity of the anthelmintic compounds is regarded as the predominant physicochemical property influencing the efficacy of cuticle passage [35]. The lipophilicity of a compound may be determined by a partitioning experiment using an organic phase (e.g., n-octanol) and water, and the subsequent quantification of the ratio of concentrations in the two phases, which is expressed by the partitioning coefficient logP. In an experimental setting with nematode larvae in an aqueous growth medium, lipophilic drugs with high partitioning coefficients preferentially distribute into hydrophobic compartments, such as the parasite's cuticular surface or lipid bilayers of cells. In contrast, hydrophilic drugs with low partition coefficients are predominantly found in hydrophilic compartments (i.e., the growth medium surrounding the worms). In order to reach its specific site of action within the nematode, a compound has to cross hydrophobic cuticle layers and cellular membranes and must therefore be able to enter and subsequently leave lipophilic bilayers [36]. In this context, it is important to stress, that the relationship between logP and permeability is not linear. By contrast, permeability decreases at both low and high logP values. It is hypothesized that these nonlinearities are due to: (i) the inability of weakly lipophilic compounds to penetrate the lipid layer of the membrane, (ii) the inability of strongly lipophilic compounds to leave the membrane, and as a result of that, (iii) the excessive partitioning of these compounds into the hydrophobic core of the membrane [37].

As an alternative to measuring logP, lipophilicity may be calculated using algorithms based on well characterized logP contributions of separate atoms, structural fragments, and intramolecular interactions amongst them [38]. This calculated logP

(or clogP) has found wide application within pharmaceutical research and is a widely accepted measure for estimating the lipophilicity of drugs, and therefore the ability of compounds to permeate cells.

Another prevalent descriptor to model compound permeability is the polar surface area (PSA) [39, 40]. In the context of this compound optimization program, PSA was defined as the sum of the van der Waals or solvent-accessible surface areas of oxygen and nitrogen atoms, including attached hydrogen atoms [41]. A compound's PSA is thus closely related to its ability to accept and donate a hydrogen, and it can be regarded as a refinement of Lipinski's N/O count [42]. Due to the great effort required to measure compound permeabilities in helminth cuticles and cellular membranes *in vivo*, values for clogP and PSA were calculated for the nematocidal and inactive compounds from the first optimization experiments.

As depicted in Figure 19.5, the most active compounds, as marked by the size of the spheres, were grouped in a sector stretching from $2 < \text{clogP} < 6$ and $70\,\text{Å}^2 < \text{PSA} < 120\,\text{Å}^2$. In this region, as indicated by the black filling of the spheres, only the entities lacking a sulfonamide functionality were found. Thus, it was reasoned that the presence of a polar sulfonamide functionality significantly contributes to the sum of polar fragments located on a molecule's surface. This, in turn, must be considered counterproductive for nematodicial activity. Accordingly, the design of the next optimization round was built on the assumption that the PSA of potentially active compounds should be less than $120\,\text{Å}^2$ and that clogP should be 2–6.

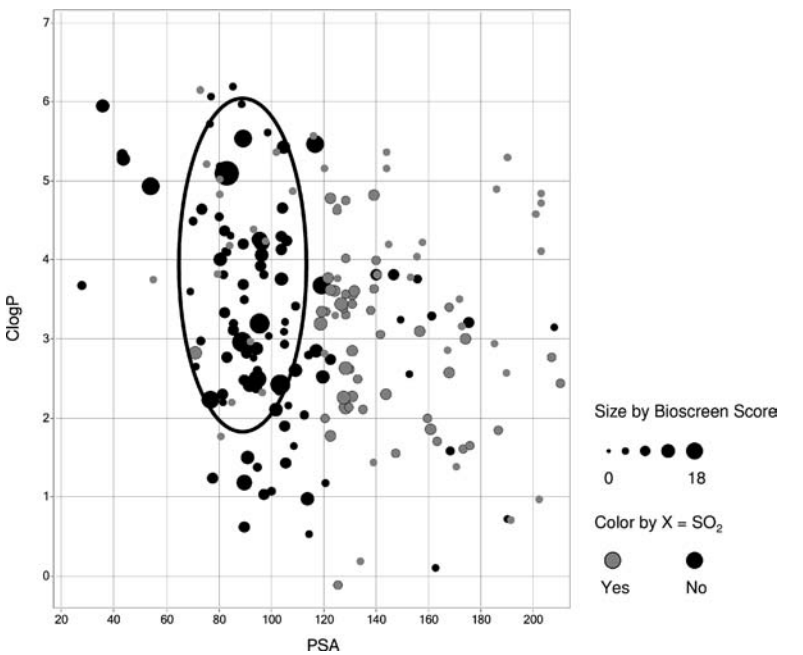
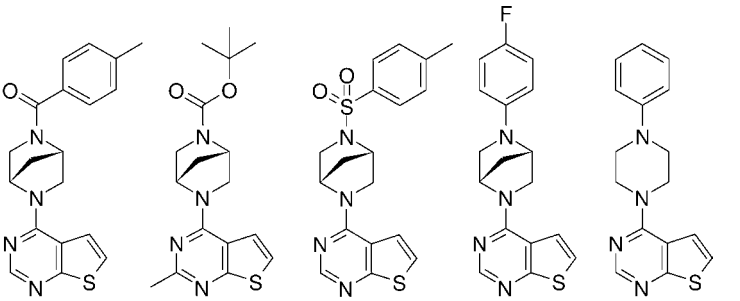


Figure 19.5 Correlation of *in vitro* activity to lipophilicity (first synthesis round).



	6	7	8	9	10
Synthesis Round	2	2	1	2	1
IC_{50} (ETC I/II *A. galli*)	39.5 nM	293 nM	3.7 nM	1.1 nM	82.1 nM
Bioscreen Score	11	14	10	17	8
PSA [Å^2]	94	75	119	81	85
clogP	2.9	3.7	3.4	4.0	3.1

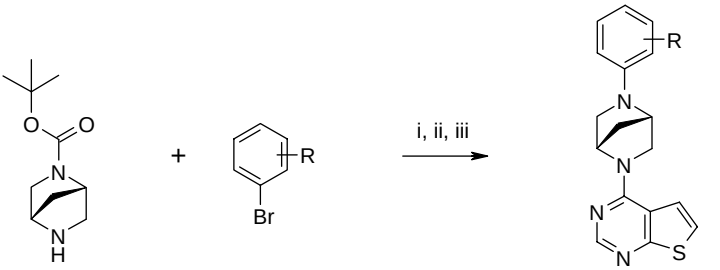
Figure 19.6 Selected examples of compounds from both synthesis rounds.

Second Optimization Cycle

Given the number of alterable positions present in the lead compound **1**, it was decided to first enumerate an all-embracing virtual library using the Legion module of SYBYL 6.8 (Tripos International, St. Louis, Miss., USA) and to subsequently narrow down on the most promising motifs. The most promising clusters according to the two selection criteria for lipophilicity were selected and representative members from each group were then synthesized and tested against ETC I/II and in nematode assays.

Figure 19.6 shows some of the compounds created during the first and second optimization rounds. Among the various motifs synthesized in the second campaign, entities carrying a diazabicyclo[2.2.1]heptane linker, like compounds **6** and **7**, were distinctly active against target complexes I/II and in the nematode assays. When compared to a compound from the first synthesis round (e.g., **8**), the negative influence of the sulfonamide functionality on nematocidal activity became apparent. On the other hand, compound **9** was considerably more active even though it has close structural similarity to the previously synthesized arylpiperazine **10**.

Encouraged by these results, a library comprising the diazabicyclo[2.2.1]heptane motif was synthesized following the general synthesis scheme (Figure 19.7). Under microwave-mediated cross-coupling conditions, commercially available *N*-boc-diazabicyclo[2.2.1]heptane **11** was reacted with a range of substituted arylbromides **12** [43]. The resulting arylpiperazines were deprotected using trifluoroacetic acid, to form amine containing building blocks, which were easily reacted with various 4-chlorothienopyrimidines to yield the desired products **13**.



11 **12** **13**

Figure 19.7 Synthetic route to diazabicyclo[2.2.1]heptane containing thienopyrimidines. Reagents and conditions: (i) $Pd_2(dba)_3$, BINAP, NaO^tBu, toluene, microwave, 120 °C; (ii) trifluoroacetic acid/dichloromethane; (iii) 4-chlorothienopyrimidine, triethylamine, *N*,*N*-dimethylformamide, or tetrahydrofuran, microwave, 110 °C.

ClogP and PSA values for these products were then calculated (Figure 19.8) and found to be within the desired range of $2 < \text{clogP} < 6$ and $70\,\text{Å}^2 < \text{PSA} < 120\,\text{Å}^2$ by majority. The bioscreen scores of these compounds were significantly improved, ranging from 15 to 18, as indicated by the size of the dark spheres.

From this optimization round, chlorobenzene **14** emerged as the most potent entity, exhibiting the best *in vitro* results. Figure 19.5 compares these results to those

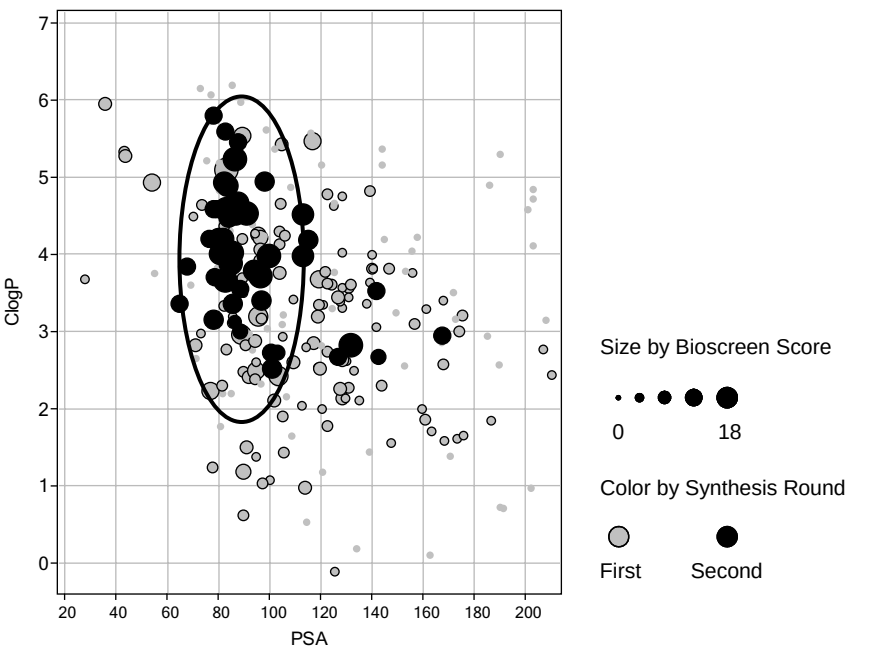
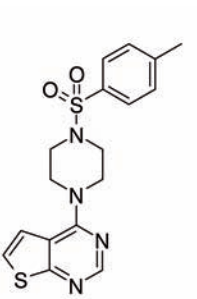
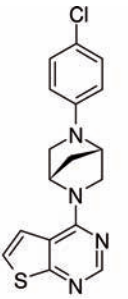
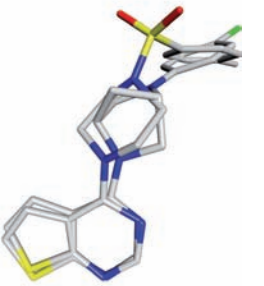


Figure 19.8 Correlation of *in vitro* activity to lipophilicity (compounds of the first and second synthesis rounds).







	1	14
IC_{50} (ETC I/II *A. galli*)	2.3 nM	0.5 nM
MEC (*A. galli* L3)	0.19 µM	0.024 µM
MEC (*O. dent.* L3)	12.5 µM	0.049 µM
MEC (*O. dent.* L4)	6.25 µM	0.012 µM
Bioscreen Score	11	18
PSA [Å²]	129	83
clogP	2.6	4.6

Figure 19.9 Structure comparison and superpositioning of lead compound 1 and the optimized compound 14. Energy calculations were performed using the MMFF94s force field [44]. The MMFF94 atomic charges were calculated based on the bond increment parameters in MMFF94 force field. To overlay the molecules, the flexible superposition tool FLEXS was applied [45]. All calculations were performed using the molecular modeling software package Sybyl 6.8 (Tripos International, St. Louis, Miss., USA).

of the initial lead compound **1**. To explain this remarkable improvement of nematocidal activity, a molecular modeling study was performed to investigate structural analogies. Hence, energy-minimized conformations of compounds **1** and **14** were generated and superimposed using the conformation of compound **1** as the rigid guiding template.

The beneficial changes from the original lead compound **1** to the optimized compound **14** could stem from the fact that the bridged piperazine mimics the angular constitution within the sulfonamide functionality. While all structural features required for inhibition of complex I in the nanomolar range were retained, the adjustment of the lipophilicity modulating properties clogP and PSA significantly improved the nematocidal activity of the compound.

Conclusion

A new class of inhibitors of the mitochondrial respiratory chain, the substituted thienopyrimidines, has been discovered on the basis of *in vitro* screens in combination

with mode of action studies. These compounds show remarkable inhibitory activity in the low nanomolar range against complex I of *A. galli* and larvicidal activity against various nematode larvae *in vitro*. Significant improvement of the initial hit compound was achieved by chemical synthesis through the adjustment of structure-inherent lipophilicity modulating physicochemical properties while retaining structural features assumed to be essential for complex I inhibition and nematocidal activity. The effectiveness of this combined optimization approach is illustrated by compound **14**, which shows significantly improved nematocidal activity compared to its predecessor, the screening hit compound **1**. This study may serve as an example for further lead optimization programs aiming at the discovery of new anthelmintic drugs.

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Part Four
Bacteria

20
Pathogenomics: Identification of Novel Drug Targets and Vaccine Candidates in Bacteria

Knut Ohlsen, Martin Eckart, Ulrich Dobrindt, Heike Bruhn, and Jörg Hacker*

Abstract

The alarming spread of antibiotic resistant bacteria has renewed the efforts to find effective strategies to combat pathogenic bacteria. Recent genome analysis of hundreds of bacterial genomes delivered intriguing insights into the composition and organization of bacterial chromosomes. Comparative genome analysis and genomic tools have been used to identify potential drug targets and vaccine candidates. This chapter reviews current progress and future prospects for exploitation of genome sequences and its impact on the development of new antibiotics and vaccines to combat re-emerging infectious disease.

Pathogenomics – Study of Pathogenesis in Genomic and Post-Genomic Era

The term "pathogenomics" refers to all aspects directly or indirectly related to pathogenic processes of microorganisms based on genome information of pathogenic and apathogenic organisms, including gene content, genomic organization, gene expression, protein expression, pathogen–host interactions, and others [1]. In the past decade, the availability of genomes from all of the important bacterial pathogens of humans, plants, and animals, as well as that of many commensal, symbiotic, and environmental microorganisms has revolutionized the study of bacterial pathogenesis. Comparative genome analysis has revealed the forces that drive pathogen evolution and has identified horizontal gene transfer and genome decay as key elements in the evolution of bacterial pathogens. Importantly, the information gained by these studies has been used both to identify new virulence mechanisms and genes involved in environmental persistence of pathogens and to understand the resistance mechanisms against antibiotics. The dream of early genome research was that the huge amount of genetic information would rapidly

lead to the development of novel strategies to combat microbial pathogens. There was great enthusiasm that the development of novel antibacterials targeting novel pathways limits resistance problems and fosters rationale vaccine design. However, this hope has not been satisfied yet. In fact, big pharma are increasingly getting out of anti-infective drug discovery, although problems due to multidrug-resistant bacterial pathogens is in some areas dramatically increasing [2].

Recent advances in genomics have provided an opportunity to expand the range of potential drug targets and have facilitated a fundamental shift from direct antimicrobial screening programs toward rational target-based strategies. The application of genome-based technologies such as expression profiling and proteomics will lead to further changes in the drug discovery paradigm by combining the strengths and advantages of both screening strategies in a single program. In addition, a major task for future work remains the identification and analysis of the large number of genes with unknown function that have been identified in all organisms sequenced to date. The complete understanding of the complex live of a microbe will necessarily lead to development of novel strategies against pathogenic microorganisms. Recent technical improvements including rapid sequence technologies, microarray analysis, gel-free proteomics, metabolome analysis, bioinformatical approaches, metagenomics, and *in vivo* technologies, when combined in systems biology approaches, are opening new avenues in antimicrobial drug research.

Bacterial Genome Information

Around 700 bacterial genomes have been sequenced and are currently available in public databases (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi) and this list will be extended by several hundred new genome sequences within the next months due to the broad availability of ultrafast sequencing technologies such as 454 (from 454 Life sciences), Solexa (from Illumina), and SOLiD (from ABI) [3]. The sequenced genomes represent more than 500 species as well as multiple strains of the same species. Starting with the deciphering of the complete genome sequence of *Haemophilus influenzae* in 1995, the genomes of all crucial pathogens have now been analyzed, such as *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Escherichia coli*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Bacillus anthracis*, *Vibrio cholerae*, *Salmonella enterica*, *Neisseria meningitides*, and others. These sequences allowed unique insights into how pathogenic bacteria are organized [4, 5]. However, the genome sequence is only the blueprint for life, and it is the coordinated expression of proteins that determines the life of an organism under different conditions, including during pathogenesis. The study of these genomes by both computational and experimental approaches has significantly advanced our understanding of the physiology and pathogenicity of many microbes and provides insights into the mechanisms and history of genome evolution [6]. Four main forces have been identified to determine genome evolution: gene gain, gene loss, mutation, and recombination. All four mechanisms are important to understand the dynamics of genome patterns of different species and clonal lineages [7–9].

Impact of Genome Research on Development of New Antibacterials

Comparative genome analysis provided novel approaches for antibacterial drug discovery in the pharmaceutical industry and academia. Importantly, the antibiotic classes currently used in clinical practice target only a limited number of cellular functions: cell wall biosynthesis, protein biosynthesis, nucleic acid metabolism, and DNA replication. Consequently, the pharmaceutical industry in the late 1990s started to transfer genome information into target identification programs to identify novel target structures in alternative cellular pathways. This strategy should lead to the identification of novel compounds that inhibit biosynthetic pathways presently not addressed by current antibacterials. The advantage of such a strategy is claimed to lie in the avoidance of resistance development and cross-resistance to convential antibiotics. This is not necessarily the fact, however, the probability that inhibition of a novel target can overcome current resistance problems is very high [10].

Identification of essential gene products

The identification of a novel target for development of new antibacterials represents a challenging task. At the beginning of the genome era, the pharmaceutical industry has used genomic information on bacteria to identify novel target structures, in particular proteins, which could be applied in screening programs for novel inhibitory compounds. In general, each essential protein may be defined as a target structure for antimicrobials. However, several further criteria have to be fulfilled before an identified protein is incorporated into target validation programs (Figure 20.1). The most important properties are:

- Selectivity (functional and/or structural difference between human and bacterial target molecules).

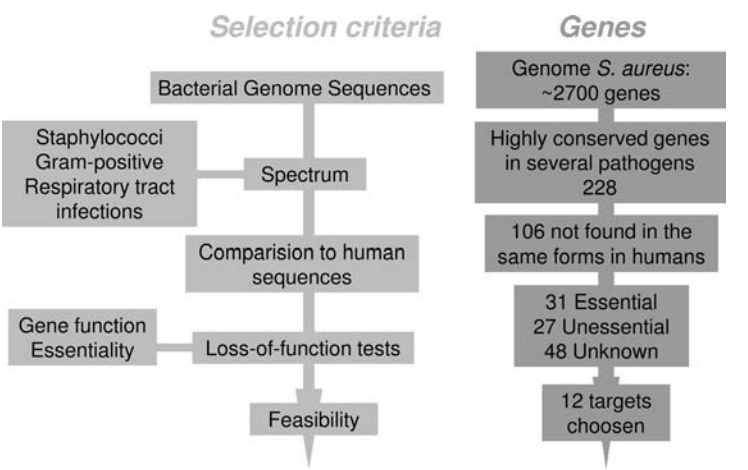

Figure 20.1 Criteria for selection of targets (example: *Staphylococcus aureus*, unpublished data).

Table 20.1 Number of essential targets in different pathogens identified in mutational studies.

Organism	Total number of genes	Number of potentially essential genes
Acinetobacter baylyi ADP1	~3300	499
Bacillus subtilis 168	4101	271
Clostridium perfringens	~3000	261
Escherichia coli K12 (MG1655)	4288	615
Escherichia coli K12B (W25 113)	4288	303
Haemophilus influenzae	~1700	642
Helicobacter pylori	~1500	344–416
Mycobacterium tuberculosis	~4000	236
Mycoplasma genitalium	484	256–350
Neisseria gonorrhoeae	~2000	219
Pseudomonas aeruginosa	~5600	343
Salmonella enteria sv. *Typhimurium* LT2	~4500	230
Staphylococcus aureus	~2700	150–658
Streptococcus pneumoniae	~2100	244

http://tubic.tju.edu.cn/deg/, http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi, [157, 158].

- Distribution (which microorganisms are susceptible).
- Assayability (can an assay for high-throughput screening be developed).
- Intracellular concentration of a target.
- Mutational potential (development of resistance).
- Bacteriocidal versus bacteriostatic action (*in vivo* efficiency) and druggability (likelihood of being able to modulate a target with a small-molecule drug).

Attempts for defining essential factors of model organisms and pathogenic bacteria have been published recently, including theoretical calculations on the minimal genome of bacteria, and extensive mutational analysis (Table 20.1) [11–13]. In the Gram-positive model organism *Bacillus subtilis* 271 genes were predicted to be essential [14]. The vast majority of these essential genes encode factors which are involved in synthesis of cell envelope, information processing, determination of cell shape and division, and generation of energy. In the important nosocomial pathogen *Staphylococcus aureus* various essential proteins have been defined by antisense RNA interference and mutagenesis studies [15, 16]. These studies provide lists of putative novel targets covering all known key pathways for example, translation, transcription, cell division, and metabolism. Most importantly, for approximately 30% of the putative essential genes the function remains unclear. Useful information on putative essential genes and phyletic patterns can be found at:

- http://www.ncbi.nlm.nih.gov/COG/
- http://web.dmz.uni-wh.de/projects/protein_chemistry/pcogr/
- http://tubic.tju.edu.cn/deg/

It is tempting to speculate that a great deal of attractive novel targets may be identified, especially within the group of proteins with unknown function. However, it has to be considered that the same gene may encode an essential function in one organism but not in another species. Recently, Zalacain *et al*. evaluated the potential of proteins of unknown function for target search by investigation of 144 orfs without significant homologies to proteins with known functions in *Streptococcus pneumoniae*. In these studies, 36 essential proteins were identified in *S. pneumoniae*. Furthermore, 14 of these factors were also essential in *S. aureus* and *Haemophilus influenzae* [17]. Importantly, nonessential *in vitro* genes were also tested *in vivo*, revealing that four out of 17 genes were also essential *in vivo*. These results clearly demonstrate that essentiality of a given gene product is not only species dependent but also dependent on the environmental test conditions. Consequently, a broader selection of bacterial targets should be applied [17]. As an important issue, target validation has to include *in vivo* test systems preferentially in different pathogens under various test conditions. Such a strategy is especially of importance for the development of broad-spectrum antibiotics.

A gene is regarded as being essential if it is not possible to inactivate this gene genetically. Therefore, gene inactivation studies are generally initiated for target-based drug discovery. Global approaches include transposon inactivation strategies. Transposons usually insert almost randomly into DNA sequences via intracellularly expressed transposases or via delivery of transposon–transposase complexes (transposomes) to the bacterial cell. In addition, transposon insertions can be initiated by *in vitro* transposition into purified chromosomes or defined chromosomal areas followed by transformation and genomic recombination. Mapping of the transposon insertion site by hybridization techniques and PCR will allow to define a list of nonessential genes. Genome-scale analysis will then identify putative essential genes. These genes can be further analyzed by site-directed recombination via single or double recombination, usually inserting marker cassettes like antibiotic resistance cassettes. The most precise way to inactivate a gene is to leave no marker sequence in the genome. Such strategies have been developed mostly for *E. coli* but also for Gram-positive pathogens such as *S. aureus* [18]. However, the fact that a gene cannot be deleted is not a final proof that a gene is essential. Only conditional mutants such as temperature-sensitive (ts) mutations or regulable gene expression systems allow the classification of a gene into essential or nonessential [19].

Several conditional expression systems have been developed to validate putative essential genes *in vitro* and *in vivo*. In most of these systems promoter elements are used that can be regulated by tetracycline, isopropyl β-D-1-thiogalactopyranoside (IPTG), arabinose, or rhamnose in Gram-negative species and by tetracycline, IPTG, xylose, fucose, cadmium, or acetamide in Gram-positives, including mycobacteria [19]. Systems using tetracycline as inducer are preferentially applied for *in vivo* gene expression as tetracycline or its nontoxic derivative anhydrotetracycline easily distributes in the body and also penetrates intracellularly [16, 20]. Recently, an alternative conditional *in vivo* expression system has been developed based on the P*spac-lacI* regulatable promoter element [21–23].

Microbial Genomics and Antimicrobial Drug Discovery

The investigation of novel targets in new pathways has been suggested to be especially promising as it is assumed that antimicrobials targeting new pathways will exhibit most likely no cross-resistance to commonly used antimicrobials and resistance development will be delayed. Genome-wide gene inactivation studies in several pathogens provided valuable information on how many potentially essential genes are encoded within the genome of pathogenic bacteria. Depending on the organisms studied and the used methods, the number of essential genes broadly ranges between 220 and 250, for example, for *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Salmonella enterica* sv. *Typhimurium*, *Streptrococcus pneumoniae*, and more than 500 for *S. aureus* (Table 20.1). However, it can be calculated that the number of putative broad-spectrum targets is probably below 100. Recently, a study dealing with *Salmonella* metabolic pathways suggested a shortage of new metabolic targets for broad-spectrum antibiotics. In a comprehensive *in vitro* and *in vivo* approach 155 promising targets to treat *Salmonella* infections have been identified, 64 of which were also conserved in other important pathogens, such as *S. aureus*, *Enterococcus faecalis*, *S. pneumoniae*, and *H. influenzae*. Almost all of these targets belong to pathways already inhibited by current antibiotics (peptidoglycan biosynthesis, folate biosynthesis, isoprenoid biosynthesis, fatty acid biosynthesis, tRNA synthases) or pathways previously considered for antimicrobial development [24]. These studies imply that, although a substantial number of proteins are conserved in relevant pathogens and are essential for bacterial growth, only a very limited number of proteins are suitable as antibacterial target. Potential targets can further be prioritized on the basis of defined criteria including essentiality, conservation in a range of pathogens, and nonexistence or low similarity in humans.

Another basic requirement of a selected target is preferentially an understanding of the function of the gene product at the level of its biochemical activity. Generally, genome sequence comparison using bioinformatic platforms conventionally allows the assignment of a function to a gene identified through DNA sequencing via similarity to a characterized protein by linear comparisons of DNA and protein sequences [25]. This approach has several limitations as it is not possible to assign the function of proteins that lack an obvious homolog. Consequently, a significant proportion of each complete genome is functionally unannotated. Recent advances in bioinformatics, however, have been applied that deduce protein function on the basis of properties other than amino acid sequence similarity [26, 27].

These methods use both theoretical prediction based on phylogeny and experimental data such as expression profiles and proteome data to identify functional linkages between proteins. The usefulness of these approaches has been demonstrated by the assignment of about half of the 2500 uncharacterized *Saccharomyces cerevisiae* proteins [28, 29]. In addition, novel software tools can be used when sequence comparisons fail to determine the function of a protein with known structure but unknown function. Direct comparison of three-dimensional (3D) protein structures is superior to simple sequence alignment, because the function of a protein is more directly a consequence of its form than its sequence. The rate of

structure determination has increased dramatically, and current structural genomics projects will impact significantly assignment of function to unknown proteins by providing sufficient information to allow other protein sequences to be modeled accurately [30–32].

Comparative genomics provides a list of potential targets with approriate bacterial spectrum and selectivity over humans. For broad-spectrum agents, bioinformatic analysis of genome sequences can be used to identify proteins that are highly conserved in a range of pathogens associated with a particular clinical indication. In addition to highly conserved targets which provide the opportunity to develop broad-spectrum antibiotics, comparison of microbial genome sequences has also revealed that a significant proportion of each genome encodes proteins that are specific to a particular pathogenic organism. The development of antibiotics with a high degree of specificity for a single organism or some related bacterial species potentially offers long-term benefits by reducing problems arising from cross resistance. Narrow-spectrum antibacterials targeting multiresitant pathogens or pathogens difficult to treat, such as MRSA, VRE, *Klebsiella* sp., *Acinetobacter* sp., *Pseudomonas aeruginosa*, or *Mycobacterium tuberculosis*, clearly have the advantage of only affecting the potential cause of an infection and not the indigenous bacteria that are essential for humans. However, there are some significant limitations to apply this strategy. First, most of the genes specific to an organism have no known or obvious function, making it difficult to progress target-based screens. Second, there are currently no rapid, accurate, and sensitive diagnostic tools to identify the specific causative agent for many infections. Third, the market potential of such a drug is low and the cost of development would be much higher than the potential sales. Although organism-specific genes may not only provide the potential targets for novel therapeutic agents but also the principal components of rapid diagnostic tools, narrow-spectrum antibacterials will only have a realistic chance for development if ultrafast diagnostic methods are introduced as routine tools in clinical practice and the costs of development will be near the expected sales [33]. Probably, public investment can help to reduce the risks for pharmaceutical companies to realize such an innovative concept of treating infection diseases that nevertheless have a high potential for being realized in the future. For example, the Bill and Melinda Gates foundation has spent more than three billion dollars to combat infectious diseases and to develop new antiinfective strategies (www.gatesfoundation.org).

Bacterial Expression Profiling for Determination and Validation of Mode of Action of Antibacterials

The availability of the complete genome sequence of an organism provides the basis to investigate the biology of that organism. However, the pure sequence provides no information on which gene is expressed under different environmental conditions or even which protein is functionally active. Transcriptional profiling using DNA microarrays represents a rapid and systematic method for the high-throughput analysis of global gene expression [34, 35].

For each individual gene mRNA concentrations can be monitored. From the perspective of drug discovery, the transcriptional patterns generated from the parallel analysis of all genes in an organism by microarray analysis can be used to unravel the function of previously uncharacterized genes for target identification, as well as for the study of cellular responses to treatments with small inhibitor molecules to determine the mechanism of action of a novel compound. Although generally applied in pharmaceutical industry for mode of action studies there are only very few publications available dealing with the transcriptional response of bacteria to novel antibacterials. To predict the mode of action of a novel antibacterial compound, a comparative global expression analysis has to be performed on the basis of diverse expression profiles. Characteristic expression profiles have then to be compared with the transcriptional response of antibiotics with known mechanisms to deduce the cellular target of the compound of interest [35, 36]. Combined with proteome data where the expression of proteins in response to antibiotics is determined, DNA microarray approaches are appropriate tools to characterize the mode of action of novel antibacterials. Bandow *et al.* investigated the effects of 30 antibacterial compounds on the *B. subtilis* proteome, while Freiberg *et al.* and Hutter *et al.* studied the genome-wide transcriptional response of *B. subtilis* to 14 and 37 antibiotics respectively [35, 37, 38]. For example, inhibitors of translational peptidyl transfer (e.g., tetracycline, chloramphenicol, erythromycin, fusidic acid) lead to the increased expression of ribosomal proteins and elongation factors. In addition, antibiotics that cause mistranslation or abort translation (e.g., aminoglycosides, puromycin), resulting in the production of defective proteins, induce chaperones and proteases. Furthermore, inhibitors that interfere with tRNA charging (e.g., mupirocin) trigger the stringent response. These examples demonstrate that it is not only possible to assign an antibiotic to inhibition of a global metabolic area, such as protein synthesis, but that further subclassification is feasible, providing first hints of the molecular target. A comprehensive list of published proteomic and transcriptomic studies for MOA determination has been produced [35, 37, 38]. For example, the novel class of phenyl-thiazolylurea-sulfonamides generate a proteomic signature indicative of a phenylalanyl-tRNA synthetase (PheRS) inhibitor. The proteome profile resembles that of mupirocin, which inhibits isoleucyl-tRNA synthetase (IleS). Both proteome signatures are mainly characterized by the induction of stringently controlled proteins. It is known that the stringent response is triggered by stress, such as amino acid starvation, which is also mimicked by aminoacyl-tRNA synthetase inhibition. A significant distinction between both compound profiles is the induction of the corresponding targets PheRS and IleS. Both target proteins were each induced more than twofold, while induction of other aminoacyl-tRNA synthetases was not observed in two-dimensional gels [39, 40]. The examples of the novel peptidyl transferase and aminoacyl-tRNA synthetase inhibitors demonstrate that novel MOAs indeed can be predicted on the basis of a compendium of expression profiles. An essential prerequisite is the fact that antibiotics with closely related MOAs are members of the reference dataset. The identification of completely novel mechanisms may remain difficult, but might be facilitated by profiles of conditional mutants [40].

These examples underscore the power of expression profiling for mode of action prediction using compendia of transcriptome and proteome profiles. The identification of completely novel mechanisms may remain difficult, but can be facilitated by inclusion of profiles derived from mutants conditionally expressing antibacterial targets. The equivalence of conditional mutant profiles and antibiotic-triggered profiles was demonstrated with a *B. subtilis* mutant downregulating the peptide deformylase. The proteome profile of this mutant correlated well with the profile of the wild type treated with the deformylase inhibitor actinonin [37]. Consequently, microarrays and proteome analysis provide vital information on the behavior of microorganisms under different conditions. Both techniques deliver complementary information to get a deeper insight into biological responses of bacterial cells to antibiotic compounds that is necessary to unravel the mode of action of a novel antibacterial. Thus, gene and protein expression profiles have a key role in antimicrobial drug discovery programs and thus significantly streamline most phases of drug discovery and development.

Target-Based Search for Novel Drugs

All of these technologies, alongside numerous other strategies, are potential tools in exploring the functionality associated with bacterial genes. An understanding of function is important to facilitate development of suitable screening assays. Although microbial genomics has revolutionized antibacterial drug discovery over recent years, only few genomics-derived compounds are currently in clinical development. What are the reasons why so many target-based screening approaches have failed to produce a sufficient number of lead compounds? Surprisingly, the success of high-throughput screenings (HTS) campaigns run by several big pharmaceutical companies was very poor due to several reasons. First, high-throughput assays based on purified enzymes yielded hits that often lack cell penetration. Second, the majority of compounds in large synthetic libraries were often too hydrophobic, too simple in structure, or lack molecular diversity to serve as optimal starting molecule for the development of antibacterials. Third, target quality was not considered adequately, as target screenability was sometimes more important defined by HTS criteria [33].

For example, GlaxoSmithKline (GSK) ran 70 HTS campaigns in the period 1995–2001, of which 16 HTS gave rise to hits, and only five of these resulted in leads. Success in finding lead compounds was only realized for peptide deformylase (PDF), enoyl-acyl carrier protein III (FabH), methionyl tRNA synthetase (MetSR) and phenylalanyl-t-RNA synthetase (PheRS) targets. Highly potent inhibitor series for MetRS and FabI with high antibacterial activity were synthesized, but these lack activity either against a suitable range of Gram-positive pathogens or against *S. pneumoniae* and Gram-negative pathogens [41]. Moreover, FabH leads were similar to known compounds and PheRS leads are still being investigated. Likewise, other companies failed to identify suitable candidates. These examples clearly demonstrate that the level of success was extremely low in relation to the large effort invested. Therefore, such programs were stopped, shifting the focus of antibacterial research

to the optimization of known antibacterial molecules, the screening of small natural product libraries, and rational design initiatives [41]. Moreover, while novel targets in already approved pathways are being investigated, the discovery of new drugs against older targets, based on new structural information, also represents a promising strategy.

Another pitfall of targets selected from comparative genomics is that validated targets are only truly validated in those particular strains for which sequence comparision was made and in which genetic knockout mutations were tested. For example, MetRS was selected as a promising target due to high sequence homology between strains of *S. pneumoniae*, *S. aureus*, and *Escherichia coli*. However, there is considerable variation in sensitivity of clinical strains of *S. pneumoniae* showing a bimodal distribution, one group being sensitive the other not. Later, the reason for this became evident, since some strains of *S. pneumoniae* express two different genes whose gene products catalyze the same tRNA aminoacylation reaction and only one enzyme was inhibited by MetRS inhibitors that were developed from HTS hits. Although genome sequences of five pneumococcal strains were available, the presence of a nonhomologous gene that encodes a protein with same activity as the target gene was unforeseeable [41]. This example impressively demonstrates the possible limitations of comparative genomics-driven target search and emphasizes genome sequencing of a large number of different clinical strains, preferentially those that are representatives of different clonal lineages.

Current implementation of ultrafast sequencing technologies will dramatically impact genome sequencing projects in the future. Hundreds of new bacterial genomes of different species will be available within a short time, providing the basis for a renewed interest in comparative genome programs which will probably deliver promising target candidates for all critical human pathogens. Although the enthusiasm of the early days of the genome era has passed, it is unquestionable that the power of genome-based technologies will significantly contribute to antimicrobial drug discovery at every stage of development. Further basic research is necessary to understand the physiology and virulence of bacterial pathogens, providing the basis for development of novel therapeutical strategies against pathogenic bacteria in the future.

Powerful Diagnostic Tools Derived from Genome Information

Bacterial infections are potentially life-threatening and require rapid identification and antibiotic susceptibility testing of the causative pathogen in order to initiate specific antimicrobial therapy. The mortality associated with invasive infections such as bloodstream infections ranges from 20 to 50%, depending on the causative pathogen and general host condition. In hospital settings, nosocomial pathogens including *Staphylococcus aureus*, *Escherichia coli*, coagulase-negative staphylococci (CoNS), *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterococcus* spp., *Streptococcus* spp., and *Enterobacter cloacae* are the most frequent etiological agents of infections. Importantly, these pathogens are increasingly resistant to a broad range of antibacterials. Thus, rapid and reliable detection of invasive pathogens, including

characterization of the causative agent to the species level and determination of its antibiotic susceptibility pattern, is crucial for appropriate therapeutical intervention. In general, routine microbiological detection of pathogens involves enrichment of the causative pathogen followed by Gram stain, subculture on agar, and subsequent biochemical identification and susceptibility testing. Definitive identification and antibiotic susceptibilities are usually not available earlier than 24–72 h. The use of automated identification systems allow us to type pathogens to the species level; however, additional strain-specific information require additional time-consuming and expensive phenotypic and genotypic tests which are not applied routinely [42].

Because the cultivation of microorganisms from blood culture or host tissues as the gold standard is slow and insufficiently sensitive when the patient has previously received antibiotics or in the presence of fastidious organisms, new molecular techniques have been developed for the detection of bacteria in clinical samples. These methods rely on DNA- or RNA-based hybridization or amplification, PCR-based detection or protein-based detection by mass spectroscopy with the aim to identify bacteria within 2 h after the first signal of growth in conventional blood cultures. The availability of complete genome sequences of all critical human pathogens has accelerated the development of rapid and sensitive microbiological diagnostics of bacterial pathogens [43].

A promising genotyping method that allows the simultaneous identification of a wide variety of genes is provided by DNA microarray technology [44]. DNA microarrays consist of hundreds of thousands of different DNA sequences spotted on a solid surface e. g. glass surface or synthesized using a photolithographic manufacturing process. These DNA sequences serve as hybridization probe forming heteroduplexes with either RNA- or genomic DNA-derived cDNA that has been labeled with a fluorescent dye. Hybridization reaction can then be detected by measuring fluorescence signals associated with each spot. There are two types of DNA microarrays; oligonucleotide-based arrays and PCR product-based arrays [44]. The major advantage of DNA microarray-based diagnostics is that in principle all relevant bacterial species including known resistance determinants can be simultaneously detected of in a single reaction. DNA microarrays have been applied successfully to the detection of bacterial species such as *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enterica*, *Yersinia* sp., *Mycobacterium tuberculosis*, *Helicobacter pylori*, and *Pseudomonas aeruginos*. Furthermore, DNA microarrays have been used to classify resistance mechanisms to β-lactams, for example, extended-spectrum β-lactamases (ESBLs), fluoroquinolones, macrolides, aminoglycosides, and others [8, 45–54].

Although highly specific, DNA microarray application to blood cultures and other clinical samples is challenging as sufficient amounts of diagnostical material has to be gained due to a relatively low sensitivity. Moreover, quality control issues have to be fulfilled before this technique can be routinely used. Most critically, high costs and technical requirements prevent broader application. In addition to DNA microarray technology, detection of microorganisms directly in blood, cerebrospinal fluid, or biopsies by pathogen-specific or broad-range PCR assays showed promising results. However, interpretation is complex, because of detection of DNA rather than living

pathogens, a risk of interfering contamination, the presence of background DNA, and the lack of an appropriate standard.

Further techniques for laboratory detection of invasive infections include fluorescent *in situ* hybridization (FISH), real-time PCR (RT-RCR), ligase chain reaction (LCR), pyrosequencing technology, terminal restriction fragment length polymorphism (RFLP), and single strand conformation polymorphism (SSCP). All of these techniques significantly benefit from complete genome sequencing projects and comparative genomics approaches. Although every technique has specific drawbacks, molecular assays are expected to replace the current conventional microbiological techniques for detection of infectious agents in patients. Patients would significantly benefit from rapid diagnosis of the causative infectious agent due to application of an appropriate therapy, including shorter course of broad-spectrum antibacterial treatment. In addition, ultrarapid, sensitive, and specific diagnosis is the prerequisite for application of narrow-spectrum antibiotics that will probably be developed to novel species specific targets in the future [55].

Future Perspectives

It has been meanwhile realized that only a very limited number of proteins have the potential for being a potent novel target for antibacterial therapies [24, 56]. The spread of antibiotic-resistant bacteria in hospitals and communities raises serious concerns. However, only a handful of new antibacterial agents have been approved for therapy since 1998, and only two of these, linezolid and daptomycin, have novel mechanisms of action, but interfer with known targets (protein synthesis, membrane). Although there is a clear need for new antibacterials, the research and development expenditures for antibacterials spent by the largest pharmaceutical companies has decreased continuously [57, 58]. The first complete bacterial genome was published in 1995, with huge enthusiasm for scientists and physicians, and a new era for antibacterial drug discovery was claimed. Now, more than 700 bacterial genomes have been sequenced and, for example, the complete genome sequences of 12 *S. aureus* strains are available in public databases. However, only a handful of targets have been selected for high-throughput screens (HTS) by genome comparision approaches to identify novel lead compounds.

Systems biology and metagenomics

Pharmacogenomics-based strategies to develop novel therapeutical strategies against antibiotic resistant pathogens may substantially benefit from the rapid progress in bioinformatics and systems biology. Several post-genomic methods provide an unprecedented richness of data on mRNA and protein expression of bacterial pathogens under different environmental conditions including during pathogenesis. In particular, detailed pictures on the transcriptome, proteome, and metabolome of different infectious agents (e.g., *S. aureus* [59], *Salmonella* [24]) have been generated. These data can now be integrated on the next level to calculations on protein–protein interactions, the formation of protein complexes, and cellular functional modules.

New insights can be gained such as large-scale interactomic maps of key host cell types [60]. Bioinformatical modeling of metabolic pathways also fosters rational design of novel targets for antibiotic therapy. Recently, such a approach has been used [24] to analyze systematically the metabolism of *Salmonella* and to deduce putative pathways for antibiotic intervention strategies. This modeling and experimental work has defined novel drug targets; however, these targets mainly reside almost exclusively in known pathways already addressed in current antibiotic therapy.

The treatment of severe infections caused by persistent bacteria is especially challenging because they survive in a dormant or low active state at body sites where antibiotics can hardly penetrate into (e.g., *Mycobaterium tuberculosis* in the lung, *S. enterica* in the gall bladder). Activation of metabolism of the dormant state could serve as a new strategy to attack these bacteria. Such a strategy is currently under investigation for treatment of *M. tuberculosis*. Comprehensive bioinformatic analysis on the pathways involved and measures to activate them and transform the dormant state into an active state should boost such efforts. If the bacteria restore active replication, they can be much more easily attacked by bacteriostatic or bacteriocidal drugs.

Currently, systems biology approaches are increasingly addressed to study complex interactions in biological systems quantitatively by the integration of data on several levels, including genomics, transcriptomics, proteomics, metabolomics, and interactomics. These approaches trigger in particular tailored strategies towards personalized and individualized medicine. The system response of the host against infections caused by bacterial pathogens can be analyzed in detail, revealing strong individual differences. In the future, this should allow to prevent severe disease outcome by prescreening of particular sensitive individuals [61, 62]. Furthermore, the response, both against the infectious agent and against the antibiotic treatment, can now be modeled using systems biology.

Immunotherapy and Vaccine Development – Alternative Strategies to Antibiotics for the Treatment of Infections by Multiresistant Bacterial Pathogens

The crisis of treatment bacterial infections due to the spread of multiple antibiotic-resistant pathogens in hospitals worldwide has renewed efforts to develop alternative antibacterial therapies. Vaccination represents (besides antibiotic therapy) the second pillar of antibacterial strategies. Highly effective vaccines are available against deadly pathogens such as *Clostridium tetanii*, *Corynebacterium diphtheria*, *Haemophilus influenzae*, *Bordetella pertussis*, subgroups of *Neisseria menigitidis*, and *Streptococcus pneumoniae*. However, vaccination against the most important nosocomial pathogens including *S. aureus*, coagulase-negative staphylococci,

enterococci, *P. aeruginosa*, *Acinetobacter baumannii*, and certain *E. coli* pathotypes as well as effective vaccines against *Mycobacterium tuberculosis*, and particular *S. pneumoniae* serotypes are lacking. Therefore, novel strategies to design effective vaccines such as genome-based vaccine design and DNA vaccination have been exploited during the past decade.

Vaccine Design in the Genomic Era – Reverse Vaccinology

In a conventional vaccinology approach, the pathogen is grown under laboratory conditions to produce specific components in a pure form and in sufficient quantity. There are some limitations of this approach as it is time-consuming, it cannot be applicated to noncultivable pathogens, and in some cases the antigens expressed *in vivo* during infections are not produced under laboratory conditions. The genomic era has open up new avenues in vaccine design, as it is now possible to compare all the genomes of pathogenic bacteria with genome sequences of apathogenic, commensal, and environmental species to define novel structures for vaccine development [63]. Bioinformatics can predict function and localization of proteins, for example, on the surface or in membranes. Recently, Rappuoli and colleagues introduced the term reverse vaccinology indicating that, in contrast to conventional vaccinology, the starting point for vaccine design is the *in silico* analysis of the genome sequences and not the live bacterium [64]. Several examples of vaccine development based on reverse vaccinology approaches have been published in recent years. The first example where comparative genome analysis was applied to identify potential vaccine candidates represents a reverse vaccinology approach against the human pathogen *Neisseria meningitides* serogroup B (MenB), the major cause of sepsis and meningitis in children and young adults. Although conjugate vaccines based on capsule polysaccharides are available against other serogroups, development of an effective vaccine against MenB constantly failed by conventional vaccinology approaches. As it was not possible to use the serogroup B capsule as vaccination structure (the capsule contains α2-8-linked N-acetylneuramic acid, which is also a common carbohydrate present in human tissues) an alternative structure had to be selected. In a reverse vaccinology approach, Rappuoli and colleagues screened the complete MenB genome sequence for vaccine candidates and predicted more than 600 novel genes that encode surface-exposed or exported proteins. These proteins were cloned and expressed in *Escherichia coli* as fusion proteins. Some 350 recombinant proteins were successfully expressed, purified, and used to immunize mice. The sera obtained were used to confirm the surface exposure of the proteins by ELISA and FACS analysis and to test for the ability to induce complement-mediated *in vitro* killing of bacteria, a test that correlates with vaccine efficacy in humans. Thus, 91 novel surface-exposed proteins were discovered, and 29 of these were able to induce bactericidal antibody response [64, 65]. In addition to the conventional outer membrane proteins with variable surface-exposed loops, many of the new proteins were lipoproteins or other types of surface-associated proteins without membrane-spanning domains. These were often conserved in sequence,

and carried multiple protective epitopes conserved in most strains. These novel proteins provide an optimal basis for the development of a novel and effective vaccine against MenB.

One problem in defining effective vaccine candidates was that bacterial antigens, including meningococcal antigens, often show considerable sequence variability. Therefore the identified antigens had to be evaluated for conservation across a panel of diverse strains of *N. meningitides* covering all common serotypes and representing the phylogeny of the species. This analysis defined several antigens which are conserved and have been shown to elicit cross-bactericidal activity against all strains tested. The most promising vaccine candidates have been tested in phase I clinical trials [66]. In a recent phase II clinical study the vaccine produced protective immune response in infants, the group of most risk. The tested vaccine formulation comprises a cocktail of the most protective antigens vaccine that showed the greatest ability to stimulate the immune system to kill bacteria from a panel of 85 strains of meningitis B representative of global and temporal diversity (Miller, *et al.*, personal communication).

The successful serogroup B *N. meningitides* example has initiated the application of the reverse vaccinology approach to other pathogens, such as *Escherichia coli*, *Streptococcus pneumoniae*, *S. agalactiae*, *Porphyromonas gingivalis*, *Chlamydia pneumoniae*, *Bacillus anthracis*, and others [67–71]. Extraintestinal pathogenic *E. coli* represents a group of facultative pathogens that cannot be easily distinguished from many commensal *E. coli*. Comparative genomic analyses have been employed to identify virulence-associated genes and genomic regions of uropathogenic *E. coli* which are absent from commensal strains [72, 73]. The search for promising vaccine targets has also recently employed comparative genomics and genome-wide analysis of gene expression *in vivo* or under *in vivo*-like conditions. Similarly, complete genome sequences of *E. coli* have been analyzed for genes that underlie positive selection in the urinary tract. Twenty-nine genes involved in virulence, cell surface structure modulation, nutrient uptake, and DNA metabolism have been extracted from the different *E. coli* genomes [74]. They may code for factors which are important for colonization or infection of the urinary tract and thus could represent interesting targets for vaccination.

Another approach is to focus on genes and their encoded products which are preferentially or even exclusively expressed in urine or during urinary tract infection (UTI). For this purpose, global transcription profiles of uropathogenic *E. coli* during UTI have been analyzed. Especially, determinants coding for fimbrial adhesins and siderophore system were found to be markedly induced upon growth in urine or in the urinary bladder. In addition to expression of these surface-associated factors, anaerobic respiration as well as the utilization of amino acids and sugar acids such as gluconate and galacturonate was increased and seemed to mirror specific metabolic requirements upon growth in the urinary tract [75, 76]. Working on the rational design of UTI vaccine candidates, outer membrane-associated protein expression during growth in human urine or during UTI of *E. coli* has been carefully investigated. These studies demonstrate that expression of various siderophore receptors is induced under these conditions and that these

proteins as well as bacterial flagellin elicit a strong antibody response in the host during urinary tract infection [77, 78].

In an effort to design a vaccine against *S. agalactiae*, also know as group B streptococcus GBS, a leading cause of newborn sepsis, the genome sequences of eight GBS isolates were analyzed to identify putative vaccine candidates. Computational algorithm analysis enabled the prediction of 312 surface-associated proteins that were cloned and tested as vaccines. It could be shown that four proteins elicited protection in mice, and an antigen cocktail of these four proteins has proven highly protective against a large panel of strains, including all circulating serotypes. Importantly, protection also correlated with antigen accessibility on the bacterial surface and with the induction of opsonophagocytic antibodies [79]. This work impressively demonstrated the power of multigenome analysis for identifying potential vaccine candidates against highly variable pathogens. A considerable novelty of this study is that none of these antigens could be classified as universal, because three of them were absent in a fraction of the tested strains, and the fourth core gene showed negligible surface accessibility in some strains. Thus, the use of multi-genome sequence information for vaccine design represented an important improvement in vaccine development in comparison to the common concept that a single genome sequence is sufficient to identify surface-associated proteins to be tested as potential vaccine candidates. Because a single genomic sequence is not sufficient to cover the variability of bacterial populations, multiple genome sequences may be necessary to identify promising vaccine candidates that are effective in species with high genome variability. As already stated in Chapter 6, current improvements of sequencing technologies are reducing the costs and increasing the speed of whole-genome sequencing. The availability of multiple genomes of a single species will significantly improve our understanding of genome organization, genome flexibility, heterogeneity, and evolution of bacterial pathogens. This will ultimately lead to the identification of novel vaccine candidates against major human pathogens.

Passive Immunoprophylaxis

The need for new therapeutic options to treat infections due to multidrug-resistant nosocomial pathogens such as *S. aureus*, coagulase-negative staphylococci, enterococci, and *P. aeruginosa* has released a number of initiatives for the development of active or passive immunotherapy approaches [80]. There are some clearly defined at-risk populations that would benefit from immunotherapeutics. Passive immunoprophylaxis using either polyclonal or monoclonal antibodies could aid many people to generate a potent immune response, for example, immunocompromised patients, premature infants, mechanically ventilated persons, and patients who carry foreign medical devices such as catheters and implants [81]. Consequently, all intensive care patients may benefit from passive immunization.

The identification of effective target structures for antibody-based therapy is a prerequisite for the development of immunotherapeutics. Promising antigens

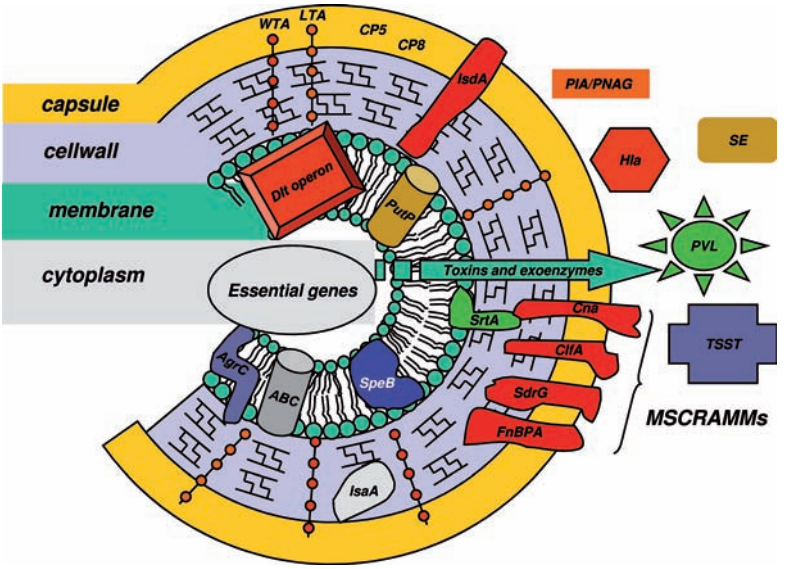

Figure 20.2 Putative novel targets for immunotherapy in *Staphylococcus aureus*. Individual components are indicated in relation to subcellular location.

can be selected from microbial surface components or bacterial toxins (Figure 20.2) [82]. Many proteins are located on the surface and play a prominent role in the process of adhesion to specific sites on human tissues or implanted medical devices [83]. Antibodies recognizing surface proteins may promote an enhanced immune clearance and may block the adherence of bacteria to tissues [84]. Several clinical trials evaluating *in vivo* efficiency of, for example, antistaphylococcal immunotherapy in human risk populations have been published; however, unfortunately, most of these studies failed (Table 20.2). For example, a hyperimmune IgG preparation to clumping factor A (ClfA) of *Staphylococcus aureus* called Veronate (Inhibitex) has been developed by collecting sera from patients with high titers to ClfA [85]. Positive phase II data had indicated the potential for protecting low birth weight infants from staphylococcal infections, but in a following phase III study Veronate failed to show efficiency [86, 87]. In another phase III clinical trial that showed no significant protection, hemodialysis patiens were vaccinated with a polysaccharide conjugate vaccine containing capsule polysaccharides type 5 and type 8 that were coupled to pseudomonas exotoxin A toxoid (StaphVAX) [88]. Furthermore, a humanized monoclonal antibody called Aurexis (tefibazumab) that targets clumping factor A is in development as an adjunctive therapy for serious *S. aureus* infections. After phase I trial in 2006, additional trials proofing efficacy are pending [89–91]. Another candidate for immunotherapy that could possibly provide protection against *S. aureus* is an immunodominant ABC transporter [92]. NeuTec Pharma has developed genetically recombinant antibodies (Aurograb) against this structure which have intrinsic

Table 20.2 Potential immunotherpeutics in development against bacterial infections.

Drug	Company	Pathogen (target)
Aurograb (phase III)	NeuTec (Novartis)	*S. aureus* (ABC transporter)
Altastaph (phase II)	Nabi	*S. aureus* (capsular polysaccharide)
Aurexis (phase II	Inhibitex	*S. aureus* (clumping factor A)
Pagibaximab (phase II)	Biosynexus and GlaxoSmithKline	*S. aureus* (lipoteichoic acid)
StaphVax (phase III)	Nabi	*S. aureus* (capsular polysaccharide)
V710 (phase II)	Intercell and Merck	*S. aureus* (conserved antigen)
ABthrax (raxibacumab) (phase III)	Human Genome Sciences	*B. anthracis* (protective antigen
Anthim (ETI-204) (phase I)	Elusys	*B. anthracis* (protective antigen)
MDX-1388 (phase II)	Medarex and MBL	*C. difficile* (toxins A and B)
ShigamAb	Caprion	Enterohaemorrhagic *E. coli* (Shiga toxins)
KB001 (phase I/II)	KaloBios	*P. aeruginosa*
KBPA101 (phaseII)	Kenta Biotech	*P. aeruginosa*

http://clinicaltrials.gov/ct2/home, [82, 93].

activity to *S. aureus*, synergistic activity with vancomycin, and a broad spectrum of activity to different strains of *S. aureus*, including the currently epidemic strains of MRSA [93]. The phase II study with Aurograb was completed in 2003 and phase III trials have commenced. Moreover, a chimeric antibody-targeting lipoteichoic acid called pagibaximab has entered phase II studies to prevent staphylococci sepsis in the very low birth weight neonate [93].

Several other experimental vaccine candidates that demonstrate some efficiency in preclinical studies are in development. These vaccines target mostly surface components such as the polysaccharide intercellular adhesin (PIA), alternatively designated poly n-acetyl-beta-1,6-glucosamine (PNAG), iron surface determinant A and B, fibrinogen-binding proteins ClfA and ClfB, and serine-aspartate (SD) repeat-containing proteins SdrD, SdrE, and SdrG [94–99]. Promising results in animal models justify ongoing clinical validation. However, the studies also reveal that, due to the great variety of virulence factors produced by *S. aureus*, a single immunologic target might not be sufficient for complete protection. Probably, a cocktail containing a combination of several staphylococcal virulence factors might be the best approach for a successful vaccine, as demonstrated recently by using a combination of IsdA, IsdB, SdrD, and SdrE antigens [98]. Despite recent progress, more experimental work is needed to develop immunotherapeutics that can protect humans at risk for serious *S. aureus* infections.

Kenta Biotech (Switzerland) is developing fully human monoclonal antibodies directed against *P. aeruginosa* that are currently being tested in clinical phase II. *P. aeruginosa* is an important Gram-negative pathogen in immunocompromised individuals such as cancer patients undergoing immunosuppressive treatment, patients with burns, and those being artificially ventilated in an intensive care unit or in

cystic fibrosis patients. Infections caused by this pathogen have an especially high mortality rate in nosocomial blood stream infections and treatment is increasingly problematic due to antibiotic-resistant isolates. Therefore, passive immunization strategies may represent an attractive supportive therapy approach.

Further, antibody therapies against *Bacillus anthracis*, targeting the protective antigen, *C. difficile*, targeting toxins A and B, and enterohemorrhagic *E. coli* (EHECs), targeting shiga toxins, are currently in development (Table 20.2). Although a clinical proof of concept is awaiting for passive immunization therapy to combat bacterial infections, the rise of antibiotic resistant bacteria urgently demands the generation of alternative treatment concepts. The current progress in post-genomic applications provides great opportunities for researchers to define novel targets for immunotherapy that may be used for the treatment of multidrug-resistant bacteria.

Antibiotics – Current Status and Future Challenges

The discovery and clinical introduction of antibiotics to treat bacterial infections belong to the most important contributions of medicine to quality of life. Antibiotics raised human life expectancy tremendously by ten years and paved the way for enormous progress in modern medicine in the field of organ transplantation, cancer chemotherapy, specific surgery procedures, and intensive care medicine. During the golden age of antibiotic discovery, in the 1940s to 1960s, almost all antibacterial classes that are now in clinical practice were introduced to treat bacterial infections, such as β-lactams, tetracyclines, chloramphenicol, aminoglycosides, macrolides, glycopeptides, streptogramines, and quinolones (Figure 20.3). However, only 12 of the new antibacterial agents have been approved for therapy since the 1990s and only two of these, linezolid and daptomycin, have novel mechanisms of action. The impressive success of antibiotics fed by a steady flow of novel and improved drugs resulted in a decline of activities in research and development for novel antibacterials. Many researchers, physicians, and authorities neglected the fact that most pathogenic bacteria evolve rapidly, being able for picking up resistance traits and adapt to a changing environment. Most importantly, medical progress itself resulted in a shift in patient populations, with a dramatic increase in the number of immunocompromised and multi-morbid patients who become infected by a range of opportunistic pathogens, including bacteria that were previously regarded as nonpathogenic, such as enterococci, coagulase-negative staphylococci, and *Acinetobacter* spp.

Nowadays, the spread of antibiotic-resistant bacteria in hospitals and communities raises serious concerns. Although there is a clear need for new antibacterials, the antibiotics research and development expenditures for the largest pharmaceutical companies are continuously decreasing [58]. The reasons for such a fatal development are manifold and include the availability of a large number of cheap, generic drugs, the complexity of drug discovery and regulatory processes, and prescription

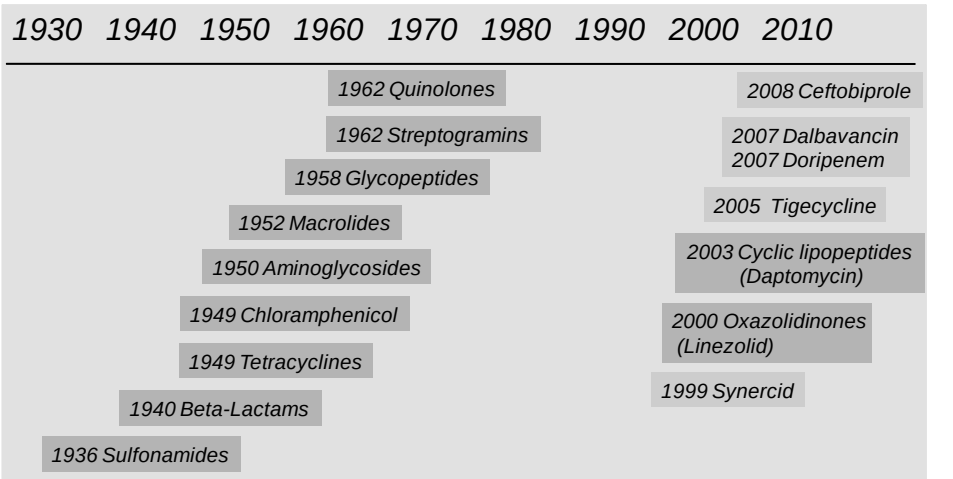

Figure 20.3 Discovery of major classes of antibiotics and novel antibiotics and introduction into the clinic. Dark boxes represent novel classes. Synercid is a streptogramin combination, tigecycline a tetracycline derivative, doripenem a carbapenem, dalbavancin a glycopeptide, and ceftobiprole a cephalosporin.

strategies where the cheapest available antibiotics are often used, even if they are inappropriate. In consequence, the current arsenal of clinically relevant antibiotics is dominated by compounds with serious resistance problems, including β-lactams, quinolones, macrolides, glycopeptides, and tetracyclines. The most problematic pathogens in terms of resistance are methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant *S. epidermidis* (MRSE), vancomycin-resistant *Enterococcus faecium* and *E. faecalis* (VRE), and multidrug-resistant *Klebsiella pneumoniae*, *Acinetobacter baumanni*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Enterobacter* species. It is very clear that, besides improved infection control measures and hygiene procedures and a more prudent use of antibiotics, novel antibiotic classes free from cross-resistance against currently used antibiotics need to be discovered to prevent a public health disaster.

Development of Novel Drugs

Where should new drugs come from? The majority of novel antibiotics that reach the marketplace are structural analogs of existing families of antibiotics (glycopeptides, quinolones, β-lactams, macrolides, tetracyclines). Novel compounds from natural and non-natural sources are usually screened in a conventional way against live multiplying bacteria [100]. However, there are some limitations of such simple growth tests and a number of upcoming alternatives provide a significant advantage. Pharmacogenomic antibiotic screens have suggested a range of promising targets. However, so far these screens have not led to the introduction of a marketable

antibiotic. In addition, nonculturable bacteria may provide an alternative source of novel antibiotics. This idea was recently spurred on by direct searches for antibiotic-producing operons in meta-genomics databanks: instead of trying to cultivate the nonculturable bacteria, their whole community is sequenced in one approach [101]. Moreover, bacteriophages have been shown to be antibacterial in animal models and may find use in specific infectious diseases.

Novel drugs against gram-positive pathogens

Current developments include a number of new agents active against gram-positive problem pathogens such as meticillin-resistant *Staphylococcus aureus* (MRSA, Figure 20.4). For example, the introduction of linezolid and daptomycin into the clinic has added two novel antibacterial classes for the treatment of infections caused by MRSA and other Gram-positive pathogens. However, there are only very few that

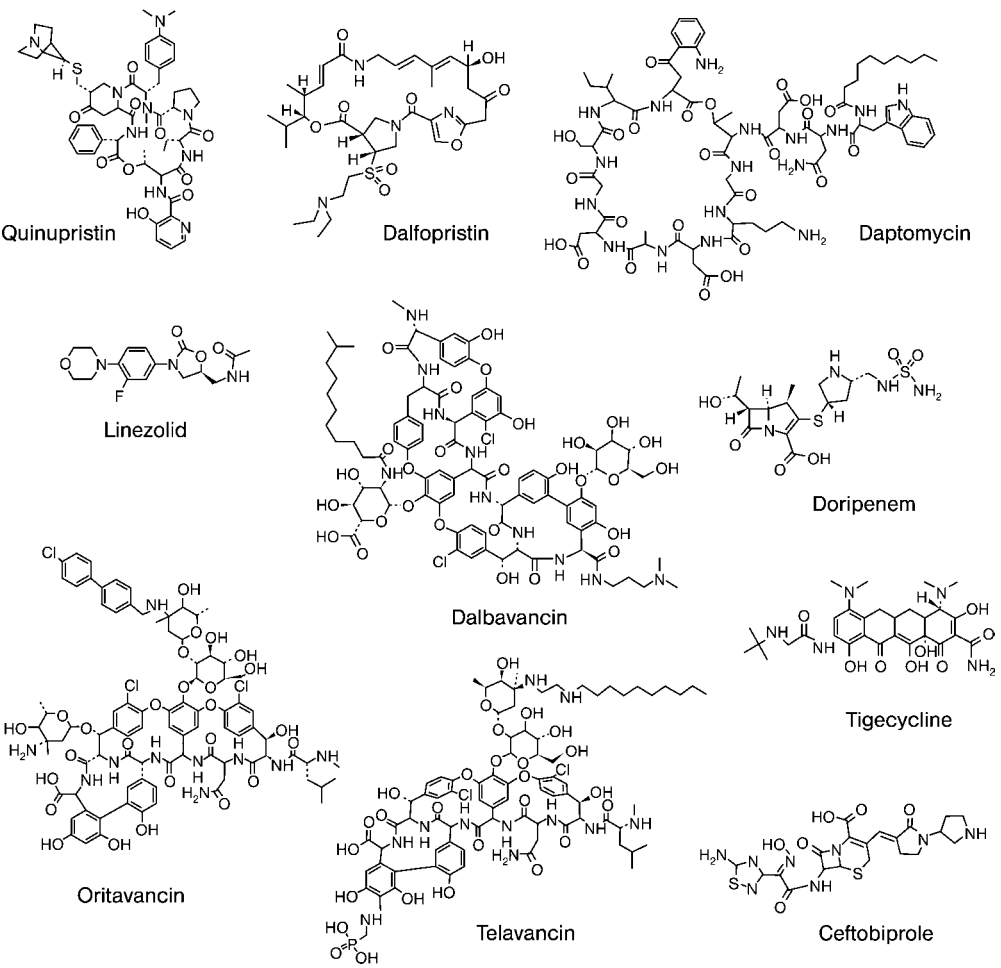

Figure 20.4 Molecular structures of novel antibiotics.

offer any advance against Gram-negative organisms [102]. The following section briefly overviews the current status of novel antibacterials against Gram-positive and Gram-negative pathogens.

Linezolid

Recently, several antibiotics have been approved or are currently in development for the treatment of serious Gram-positive infections [103]. Probably, linezolid (Zyvox, ZyvoxID; Pfizer Inc., New York, N.Y., USA) represents the most promising novel drug launched recently to treat infections due to multidrug-resistant Gram-positive bacteria. Linezolid, a completely synthetic oxazolidinone, inhibits the initiation of protein synthesis at the 50S ribosome [104, 105]. Although, linezolid blocks protein synthesis like many other antibiotics, such as macrolides, aminoglycosides, tetracyclines, streptogramines, chloramphenicol, and lincosamides, the mode of action of linezolid is unique as the drug inhibits protein synthesis at a very early phase. Binding of linezolid to the 50S subunit of the ribosome prevents assembly of the ribosome and thereby formation of a functional initiation complex consisting on ribosome, tRNA, and mRNA [104]. Based on this unique mode of action, cross-resistance to currently used antibiotics has not been observed and will probably not emerge. Linezolid has a broad activity against many Gram-positive pathogens, including MRSA, methicillin-resistant *S. epidermids* (MRSE) and other coagulase-negative staphylococci, vancomycin-resistant *S. aureus* (VRSA), glycopeptide intermediate-resistant*S.aureus*(GISA),penicillin-resistantstreptococci,vancomycin-resistant enterococci, *Listeria monocytogenes*, *Bacillus fragilis*, *Clostridium perfringens*, and others. Linezolid is approved for the treatment of nosocomial pneumonia, complicated skin and skin structure infections, bacteremia, and also necrotizing infections [106, 107]. One major advantage of linezolid is that the drug can be given orally. Several studies compared the efficiency of linezolid treatment with vancomycin. In general, most of these studies found that linezolid treatment is associated with significantly higher clinical cure rates and reduced length of hospitalization. Importantly, linezolid treatment of patients with skin infections, fasciitis, and pneumonia due to community-acquired MRSA (in particular USA300 strain) may be superior to other therapies. The severity of these infections is associated with production of exotoxins, especially Panton–Valentine leukocidin (PVL), and it has been demonstrated that linezolid reduces significantly and specifically the production of exotoxins, such as PVL, α-toxin, and toxic-shock syndrome toxin-1 [108]. Resistance to linezolid is extremely rare but, however, may increase with broad application over time [109–111]. In one study, only eight linezolid-resistant isolates were detected from 9833 Gram-positive cocci [112]. More recently, several reports documented that activity of linezolid against MRSA, MSSA, MRSE, MSSE, and streptococci remains very high (>99%). In contrast, linezolid resistance among enterococci is in general more common [113]. *In vitro* development of resistance occurs very slowly with serial passage, but when it does occur, it is related to point mutations at G2576T, G2447U, or T2500A of the 23S rRNA genes [104, 109, 110]. *In vivo* only the G2576T mutation is of clinical relevance. As all targeted pathogens harbor several copies of the 23S rRNA

(enterococci six, *S. aureus* five) the level of resistance may be correlated to the number of 23S rRNA genes that are mutated. Despite the advantage of linezolid in the treatment of infections caused by Gram-positive multidrug-resistant pathogens, there are some reports on safety concerns. In particular, linezolid treatment is associated with serotonin toxicity, especially in patients that received concomitant treatment with a selective serotonin reuptake inhibitor, and thrombocytopenia. Overall, linezolid represents an important treatment option of severe infections caused by multidrug-resistant Gram-positive pathogens. Recently, new oxazolidinones called the Rx-01 family were developed with activity against linezolid resistant enterococci and streptococci and activity against *Haemophilus influenzae* and *Moraxella catarrhalis* [114].

Daptomycin

Daptomycin (Cubicin; Cubist Pharmaceuticals, Lexington, Mass., USA) is a cyclic lipopeptide that was approved in 2003 for use in soft tissue infections, bacteremia, and right-sided endocarditis, but not for treatment of pneumonia due to inhibition of the drug by pulmonary surfactant [115, 116]. Daptomycin has an unique mode of action, killing bacteria in a concentration-dependent manner by binding preferentially to Gram-positive bacterial membranes. Insertion into the membrane causes rapid membrane depolarization and bacterial cell death due to disruption of critical metabolic functions, such as protein, DNA, and RNA synthesis. [117]. *In vitro* studies demonstrated that daptomycin had bactericidal activity equal to or greater than that of vancomycin, linezolid, and quinupristin-dalfopristin [118]. Several mechanisms of action are known, such as perturbation of calcium-dependent channels to disrupt the cytoplasmic membrane, probably by altering the membrane potential [119]. Daptomycin is bactericidal against MRSA, MRSE, and VRE, inclusive linezolid-resistant isolates. Clinical studies revealed that the difference between daptomycin treatment and standard therapy of MRSA was not statistically significant. Resistance development under therapy has been reported, and it can also be achieved in the laboratory with serial passages due to mutations in MprF, a lysylphosphatidylglycerol synthase, *yycF*, *rpoB*, and *rpoC* [120]. Clinically, treatment failure has been associated with increasing MICs, as documented in individual case studies and a randomized study of *S. aureus* bacteremia and endocarditis [117]. More data are needed, but there is clinical and microbiological evidence that daptotomycin can be used as alternative option to vancomycin in the management of MRSA bacteremia. Although clinicians should monitor isolates for resistance to daptomycin, the available data support its use in the treatment of MRSA bacteremia and endocarditis, both as initial and salvage therapy.

Glycopeptides

Several semisynthetic glycopeptide antibiotics, for example, dalbavancin, telavancin, and oritavancin have been developed as an alternative to the naturally available glycopeptides, vancomycin and teicoplanin. These compounds have been demonstrated to be more active than vancomycin or teicoplanin against Gram-positive bacteria. Dalbavancin (Zeven, Pfizer Inc., New York, N.Y., USA) is a second-generation bactericidal lipoglycopeptide that has been approved by the

FDA in 2007 for treatment of patients with complicated skin and skin structure infections (SSSIs), including those caused by methicillin-resistant MRSA. Dalbavancin was chemically derived from a naturally occurring teicoplanin-like glycopeptide produced by the actinomycete *Nonomuria* spp. Modifications of the parent compound included derivatization of functional groups such as the C-terminus and N-terminus of the peptide, removal of sugars, and addition of acyl moieties. Like other glycopeptides, dalbavancin forms a complex with the C-terminal D-alanyl-D-alanine of growing peptidoglycan chains, thereby inhibiting bacterial cell wall biosynthesis [121]. In addition, dalbavancin is able to anchor its lipophilic side-chain in the bacterial membranes, increasing the affinity of dalbavancin for its target and to increase its antimicrobial potency. Consequently, dalbavancin possesses more potent *in vitro* bactericidal activity than vancomycin or teicoplanin against many resistant Gram-positive organisms, such as MRSA [122]. Dalbavancin has also been shown to be active against one of the VRSA strains isolated in the United States (MIC: 0.5 μg/ml). Dalbavancin also inhibits vancomycin-susceptible and -resistant enterococcal strains, (MIC range: 0.03–0.12 μg/ml), but has poor activity against vancomycin-resistant (*vanA*) enterococci (MIC_{90}: 32 to >128 μg/ml). This lack of activity against VRE strains that contain the *vanA* gene differentiates dalbavancin from the other investigational glycopeptides, oritavancin and telavancin. Oritavancin and telavancin have a second mechanism of action, the transglycosylation of the peptidoglycan, which appears to explain their activity against *vanA* expressing VRE. Dalbavancin has an extended serum elimination half-life (6–10 days), which allows for once weekly dosing. In several studies dalbavancin was comparable in spectrum, but superior in potency to vancomycin against staphylococci [123]. Dalbavancin resistance has not been reported yet. In conclusion, dalbavancin exhibited greater potency than vancomycin and teicoplanin or lipopeptides, streptogramin combinations, and oxazolidinones against Gram-positive pathogens associated with SSSI or catheter-related blood stream infections, and with its unique pharmacokinetic profile, ease of use, and good safety profile, the drug is a valuable alternative in the treatment of infections caused by Gram-positive cocci [122].

Telavancin (TD-6424; Theravance, South San Francisco, Calif., USA), is another semisynthetic lipoglycopeptide that targets cell wall synthesis and disruption of membrane barrier function in Gram-positive pathogens, including streptococci, [124, 125]. Unlike dalbavancin, telavancin is also active against *vanA*-containing enterococci (MIC_{90}: 4–16 mg/L). The half-life of the drug is 7–9 h, allowing once daily dosing. Phase 3 results indicate higher clinical cure rates associated with telavancin than with vancomycin in SSSI patients infected with MRSA. The drug is awaiting approval by the FDA [106].

Oritavancin (Targanta Therapeutics, Cambridge, Mass., USA) is also a semisynthetic glycopeptide in development that targets vancomycin-resistant staphylococci and enterococci. The compound has a long half-life of 100 h and is being tested in patients with complicated SSTIs, catheter-related bloodstream infections, and nosocomial pneumonia [106, 126].

Quinupristin/Dalfopristin

Quinupristin/dalfopristin (Synercid; Monarch Pharmaceuticals Inc., Bristol, Tenn., USA) is a combination of two semisynthetic pristinamycin derivatives, quinupristin and dalfopristin, in a 30 : 70 (w/w) ratio, being the first intravenous streptogramin. It belongs to the macrolide/lincosamide/streptogramin group of antibiotics that exerts potent *in vitro* activity against the Gram-positive organisms most frequently encountered in complicated skin and skin structure infections, including *S. aureus* and *S. pyogenes*. In addition, quinupristin/dalfopristin has activity against most methicillin-, lincosamide-, and erythromycin-resistant strains of coagulase-negative staphylococci and *S. aureus*. The drug is also active against glycopeptide-resistant (vancomycin- and teicoplanin-resistant) *S. aureus* and vancomycin-resistant *Enterococcus faecium*, but not against vancomycin-resistant *E. faecalis*. Moreover, quinupristin/dalfopristin is not active *in vitro* against Gram-negative enteric bacilli or *Pseudomonas aeruginosa*. Cross-resistance has not been reported between quinupristin/dalfopristin and glycopeptide, quinolone or β-lactam antimicrobials. Synercid was approved by the FDA in 1999 to be the first antibacterial drug to treat infections associated with vancomycin-resistant *E. faecium* bacteremia (VREF) when no alternative treatment is available and also for complicated skin and skin structure infections. In addition, clinical indications of quinupristin/dalfopristin include intra-abdominal infections, bacteremia, and urinary tract infections. Quinupristin and dalfopristin act on the bacterial ribosome. Dalfopristin has been shown to inhibit the early phase of protein synthesis while quinupristin inhibits the late phase of protein synthesis. *In vitro* drug interaction studies have demonstrated that synercid significantly inhibits cytochrome P450 3A4 metabolism of cyclosporin A, midazolam, nifedipine, and terfenadine. Therefore, coadministration of synercid with drugs which are cytochrome P450 3A4 substrates and possess a narrow therapeutic window requires caution and monitoring of these drugs (e.g., cyclosporine). In several clinical trials, quinupristin/dalfopristin was compared with vancomycin to treat infections caused by MRSA. In therapy of SSSIs and hospital-aquired pneumonia, the clinical success rate of quinupristin/dalfopristin was comparable to the comparator substance. However, a higher incidence of drug-related adverse events occurred with quinupristin/dalfopristin as compared to other agents [115, 127]. Resistance development has been reported, but in general resistance to streptogramins is not very frequent in *E. faecium* of human origin and staphylococci. Interestingly, the streptogramin combination virginiamycin was used for more than 20 years as a growth promoter in commercial animal husbandry in several European countries and in Northern America, creating a reservoir for streptogramin-resistant *E. faecium*. In the majority, streptogramin resistance in *E. faecium* (and related species) is encoded by the resistance determinants *vatD* and *vatE* (formerly named *satA* and *satG*, respectively). However, there are also quinupristin/dalfopristin-resistant strains that do not possess these genes. Streptogramin resistance genes are probably transferred by conjugative plasmids as shown by *in vitro* filter matings [128, 129].

Novel Broad-Spectrum Antibacterials

Tigecycline

Tigecycline (Tygacil; Wyeth Pharmaceuticals Inc., Madison, N.J., USA) is a semisynthetic derivative of minocycline with a glycylamido moiety attached at the 9 position of the D-ring of the base molecule. It is the first clinically available drug in the class of glycylcyclines. The drug exhibits broad-spectrum activity against most of the Gram-positive and Gram-negative pathogens including MRSA, penicillin-resistant *S. pneumoniae*, beta-hemolytic streptococci, vancomycin-susceptible enterococci, Enterobacteriaceae (including extended-spectrum beta-lactamase isolates), *Acinetobacter* spp., and anaerobes (*Bacteroides* spp., peptostreptococci, *Clostridium perfringens*). Although tigecycline is structurally similar to tetracyclines, it is not a substrate for TetA-E efflux pumps, and therefore is also effective against most Enterobacteriaceae and *A. baumannii* with acquired tetracycline resistance. However, tigecycline does not exhibit potent antibacterial activity against *P. aeruginosa* since it is substrate of MexXY efflux pumps. Tigecycline inhibits protein synthesis in a broad range of bacteria by binding to the 30S ribosomal subunit with fivefold higher affinity than tetracycline that blocks entry of amino-acyl tRNA molecules into the A site of the ribosome. This prevents incorporation of amino acid residues into elongating peptide chains, inhibiting protein synthesis and bacterial growth across a broad spectrum of pathogens. It is approved for the treatment of skin and soft tissue infections, and intra-abdominal infections and is currently being evaluated for the treatment of hospital and community-acquired pneumonia [106, 130, 131]. In a recent study, tigecycline activity was evaluated against 11 808 pathogens isolated from 30 medical centers in the United States. Tigecycline was highly active against *S. aureus* and coagulase-negative staphylococci, regardless of oxacillin resistance, was eight- to 16-fold more potent than linezolid against enterococci, and was the most potent agent tested versus *Streptococcus pneumoniae*. Against Enterobacteriaceae, tigecycline was as active as imipenem. In that study tigecycline resistance was rare (0.3%), demonstrating the high potency of tigecycline against clinically relevant Gram-positive and Gram-negative pathogens [132]. Tigecycline represents the only new agent launched recently that is capable of circumventing extant resistance problems due to multidrug-resistant Enterobacteriaceae and *Acinetobacter baumannii*.

Ceftobiprole

Ceftobiprole (Zeftera; Basilea, Basel, Switzerland; in cooperation with Johnson & Johnson, Raritan, N.J., USA) is a broad-spectrum cephalosporin with demonstrated *in vitro* activity against Gram-positive cocci, including MRSA and MRSE, penicillin-resistant *S. pneumoniae*, *Enterococcus faecalis*, Gram-negative bacilli including AmpC-producing *Escherichia coli* and *Pseudomonas aeruginosa*, but excluding extended-spectrum beta-lactamase-producing strains. Like cefotaxime, ceftriaxone, ceftazidime, and cefepime, ceftobiprole possesses limited activity against anaerobes such as *Bacteroides fragilis* and non-*fragilis Bacteroides* spp. In phase III trials patients with Gram-positive skin infections were treated with either ceftobiprole or vancomycin. No difference in clinical cure was reported between the two drugs. Ceftobiprole has

so far demonstrated a good safety profile in preliminary studies with similar tolerability to comparators [133, 134]. Currently, ceftobiprole has completed phase III trials for complicated skin and skin structure infections due to MRSA and nosocomial pneumonia due to suspected or proven MRSA; phase III trials are also ongoing in community-acquired pneumonia. Ceftobiprole has obtained regulatory approval in Canada in 2008 and is awaiting a decision by the FDA. *In vitro* resistance development studies indicated that ceftobiprole has a low propensity to select for resistant subpopulations. Ceftobiprole, like cefepime, is a weak inducer and a poor substrate for AmpC beta-lactamases. Ceftobiprole has a modest post-antibiotic effect (PAE) of approximately 0.5 h for MRSA and a longer PAE of approximately 2.0 h for penicillin-resistant pneumococci. The broad-spectrum activity of ceftobiprole may allow it to be used as monotherapy in situations where a combination of antibacterials might be required. Furthermore, being a compound derived from an established class, ceftobiprole represents a safe alternative to more novel compounds. Further clinical studies are needed to determine the efficacy and safety of ceftobiprole and to define its role in patient care [133].

Doripenem

Doripenem (Doribax; Johnson & Johnson, Raritan, N.J., USA) is a broad-spectrum carbapenem antibiotic that was approved by the FDA in 2007 for the treatment of complicated urinary tract and intra-abdominal infections. Doripenem has been shown to be active against several strains of Gram-positive and Gram-negative bacteria, being particularly active against *Pseudomonas aeruginosa* [135]. The drug has a number of features, being an useful addition to currently used carbapenems such as enhanced activity against nonfermentative Gram-negative bacilli, bactericidal activity against most pathogens, stability to human renal dehydropeptidases, stability to common bacterial β-lactamases, including extended-spectrum β-lactamases (ESBLs), post-antibiotic effects against *P. aeruginosa*, potent activity against penicillin-resistant streptococci, and is effective at low doses [136, 137].

Several other novel antibiotics are in clinical development, including the broad-spectrum cephalosporin ceftaroline, carbapenems like ertapenem, oral tomopenem, faropenem medoxomil, and PF-3 709 270 (Pfizer, in phase I), the trimethoprime-related drug iclaprim, a diaminopyrimidine that inhibits dihydrofolate reductase, and quinolones such as garenoxacin, sitafloxacin, and DX-619 [106, 138].

Novel Targets and Old Pathways

As already stated, a great deal of discovery work has been performed to identify novel targets in biochemical pathways that are not currently targeted by antibacterials. The challenge of this task is mirrored by the fact that no new antibiotic that targets a novel pathway will reach the clinics by 2010 or even 2015. Thus, renewed interest is directed towards the discovery of new compounds against old targets or already addressed pathways. Here, for example new structural data of target proteins will help to define novel inhibitors based on computer-aided modeling. The following sections briefly

discuss selected examples of drugs inhibiting novel targets as well as new compounds targeting old pathways.

Cell division (FtsZ)

FtsZ is a tubulin-like GTPase which plays an essential role in bacterial cell division. Recently, a compound named viriditoxin was identified that blocked FtsZ polymerization. Viriditoxin exhibited broad-spectrum antibacterial activity against clinically relevant Gram-positive pathogens, including methicillin-resistant *S. aureus* and vancomycin-resistant *Enterococci*, without affecting the viability of eukaryotic cells [139].

Deformylase (Pdf1)

Peptide deformylase (PDF) is an essential bacterial metalloenzyme which deformylates the *N*-formylmethionine of newly synthesized polypeptides. PDF is essential for bacterial growth and represents an attractive novel target [140, 141]. The gene encoding PDF (*def*) is present in all sequenced pathogenic bacterial genomes. The X-ray structure of PDF has been resolved, defining PDF as a new class of metalloproteases [142]. Several hydroxamic acid derivatives have been reported to be potent PDF inhibitors with *in vitro* antibacterial activity [143, 144]. Importantly, one compound termed BB-83 698 was also active in systemic models of *S. aureus* infection in the mouse and represents the first compound in this novel class to enter clinical trials [145].

Type II fatty acid synthesis pathway

Type II fatty acid synthesis is essential for bacterial cell viability. In bacteria, FabH, FabG, FabF/B, FabA/Z, FabI, and FabF are involved to synthesize β-ketoacyl-ACP. Several inhibitors of this pathway are known, including the antiseptic triclosan targeting FabI, isoniazid which also targets this enzyme in *Mycobacterium tuberculosis*, and cerulenin which is a selective FabF/B inhibitor. Novel compounds have been isolated, including platencin and platensimycin acting on FabF/B (platensimycin) or FabF/B and FabH (platencin), or naphthyridin acrylamide derivatives targeting FabI [146–148]. The main problem with FabI inhibitors is the lack of broad-spectrum activity as, for example, *S. pneumoniae* possesses FabK as enoyl-ACP reductase [149]. Nevertheless, potent FabI inhibitors may be used against staphylococci. The recently described FabF and FabF/FabH inhibitors platensimycin and platensin, isolated from *Streptomyces platensis*, are promising candidates for novel antibacterial drugs, as these compounds show broad-spectrum Gram-positive activity, and importantly, have been proved for *in vivo* efficacy [147, 148].

Cell wall synthesis

Novel effective compounds against targets of the cell wall machinery are excellent candidates for new antibacterials. Several potential targets of cell wall synthesis pathways have been postulated, including penicillin-binding proteins (PBPs), UDP-N-acetylglucosamine-enolpyruvyl reductase (MurB), UDP-N-acetylglucosamine-enolpyruvyl transferase (MurA), UDP-N-acetyl muramyl L-alanine ligase (MurC),

N-acetylglucosamine-1-P acetyl transferase (GlmU), and D-alanine:D-alanine ligase (Ddl).

There are several compounds in preclinical and clinical development targeting lipid II, including glycopeptides, lantibiotics (e.g., nisin, mersacidin), mannopeptimycins, and ramoplanin [150]. Lipid II is a membrane-anchored cell wall precursor that is essential for bacterial cell wall biosynthesis. The effectiveness of inhibiting lipid II-derived pathways as an antibacterial strategy is highlighted by the fact that this molecule is the target for at least four different classes of antibiotics, including the clinically important glycopeptide antibiotic vancomycin. The spectrum of activity of novel lipid II inhibitors encompasses mostly Gram-positive pathogens, including problematic resistant types like MRSA, VRSA, vancomycin-resistant enterococci (VRE), and pneumococci. Structural modifications of peptide antibiotics increased also activity against some Gram-negative bacteria, such as *Helicobacter pylori*, *Campylobacter jejuni*, *Neisseria* spp., *Salmonella* spp., *Pseudomonas aeruginosa*, and *Shigella* spp. strains.

t-RNA synthetases

t-RNA synthetases meet many of the criteria for antibacterial targets. These enzymes are essential for growth of all pathogenic species and catalyze a limiting step in a vital bacterial function. Several aminoacyl-tRNA synthetases have been validated as potential drug targets, and lead compounds with potent inhibitory activities have been generated to block, for example, Ile-, Phe-, Met-, Tyr-, Pro-, and Trp-tRNA synthetase [39, 41, 151]. Although, theoretically all 20 aa-t-RNA-synthetases represents attractive targets and a large number of promising lead compounds have been identified, only a few agents have progressed into clinical development [41, 152]. Mupirocin is the only example of an inhibitor of aa-tRNA-synthetases in clinical use. The drug is a potent inhibitor of isoleucyl-tRNA synthetase and has a specific importance for control of MRSA [153]. This topical antibiotic, mostly used as a cream to eradicate MRSA from the nose, is highly active against *S. aureus* and *Streptococcus pyogenes*, also active against *H. influenzae*, *Neisseria* spp., and *Bordetella pertussis*, but relatively inactive against anaerobic streptococci, enterococci, and most Gram-negative bacteria [151]. Phenyl-thiazolylurea-sulfonamides were identified as a class of inhibitors of phenylalanyl (Phe)-tRNA synthetase by high-throughput screening and chemical variation of the lead structures. The compounds inhibit Phe-RS of *Escherichia coli*, *H. influenzae*, *S. pneumoniae*, and *S. aureus*, with 50% inhibitory concentrations in the nanomolar range [39]. Further studies are needed to develop active compounds that act also systematically against *S. aureus* and other pathogens.

Bacterial DNA gyrase and topoisomerase

Bacterial DNA gyrase and topoisomerase IV (topo IV) are highly conserved type II topoisomerases that play essential roles in promoting DNA replication and transcription. These molecules are attractive targets for antibacterial drug discovery. The fluoroquinolones binds to the GyrA subunit of gyrase and/or the ParC subunit of topoisomerase IV and blocks essential functions. The essentiality and evolutionary

conservation of gyrase and topoisomerase IV in bacteria suggest broad-spectrum antibacterial activity to the fluoroquinolones. A recent advance was obtained by a structure-guided drug design approach to optimize a novel series of aminobenzimidazoles that inhibit the essential ATPase activities of bacterial DNA gyrase and topoisomerase IV [154, 155]. These studies led to the development of compounds with potent activities against a variety of bacterial pathogens, for example, *S. aureus*, *H. influenzae*, *S. pneumoniae*, *E. coli*, and *E. faecalis*.

Efflux pumps

Inhibition of intracellular efflux pumps have been largely the research focus in multidrug-resistant (MDR) microorganisms. In Gram-negative bacteria efflux is a major resistance mechanism, as simply upregulation of endogenous efflux pumps prevents the antibiotic from reaching their intracellular target. In particular, efflux is a central element of resistance in *P. aeruginosa* and *A. baumannii*. Therefore, identifying compounds capable of selectively interfering with bacterial multidrug efflux pumps would provide an extremely useful therapeutic development. That inhibition of prokaryotic efflux pumps can be achieved was demonstrated with MC-2007 110 and derivatives which inhibited multiple pumps of *P. aeruginosa*; however, these compounds have not been further evaluated in clinical trials. Only one compound, EPI (MP-601 205: Mpex Pharmaceuticals), has entered clinical phase I studies for the treatment of *P. aeruginosa* infections in cystic fibrosis patients. These examples demonstrate that more basic research is needed to identify novel lead candidates that target efflux pumps in multidrug-resistant pathogens [138].

Conclusion

The global spread of antiobiotic resistant pathogenic bacteria demands urgently an orchestrated action on several levels of research, development, clinical application, and authority. Antimicrobial therapy alone is often inefficient in terms of controlling antimicrobial resistance [156]. Multifaceted intervention seems to be the most efficient method to control the spread of multidrug-resistant organisms. Longer term, we need not only to foster antibacterial drug discovery and improved therapeutic regimen but also to complement this strategy with better monitoring of bacterial resistance and antibiotic use, implementation of stringent hygiene guidelines and infection control measures, improved educational programs and rotation policies, rapid diagnostic procedures, and personalized medicine. Furthermore, a prudent use of existing antibiotics (avoidance of unnecessary prescriptions, improved dosage regimens) is important and will help to address the resistance problems. There is good reason to believe that future antibiotic research will be successful, as never before have we had such a profound knowledge on bacterial physiology, virulence, and resistance mechanisms. Current progress in genomics and especially post-genomics will definitely lead to the identification of novel targets

in new pathways. However, there is more basic research necessary to unravel the functions of unknown proteins, as within this group of molecules several promising targets are waiting for their discovery.

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21
Tuberculosis Drug Discovery: Issues, Gaps and the Way Forward

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Abstract

There is an urgent need for new antituberculosis (antiTB) drugs. Currently, six to nine months of chemotherapy are required to achieve cure of the disease. The existing front line antimycobacterials are slow-acting, and as a consequence, the incidence of drug resistance is increasing. Our second line drugs are even less efficacious. Here we discuss two major issues in TB drug discovery. First, how can attractive leads be identified, leads that have whole-cell activity? This is a fundamental problem in antibacterial drug discovery and not specific to TB. Second, we address issues that are disease-specific: how can we identify development compounds that have the potential to be drastically more efficacious against tuberculosis; and is our cell assay, is our animal model predictive for efficacy in man? We address questions around the disconnect between animal models and human disease pathology, issues of lesion-specific drug-tolerant bacterial subpopulations, and lesion-specific drug penetration.

Tuberculosis and the Objectives for TB Drug Discovery

Tuberculosis (TB), caused by the tubercle bacillus *Mycobacterium tuberculosis* (MTB), is a global health emergency (Figure 21.1). The disease claims a life every 10 seconds. Two factors, persistence and resistance, are making it difficult to bring the epidemic under control [1]. The term "persistence" describes the survival of MTB in human lesions despite extended therapy with our best current drugs. It is not clear whether the long treatment required to achieve cure is due to altered, drug-tolerant, physiological states of bacilli subpopulations residing in the various lesions, or whether the drugs cannot penetrate lesions effectively, or both. Currently, even the most effective regimen requires four drugs (isoniazid, rifampicin, pyrazinamide, ethambutol) and at least six months of therapy [2, 3]. As patients feel better within a few weeks, well enough to want to discontinue their medication,

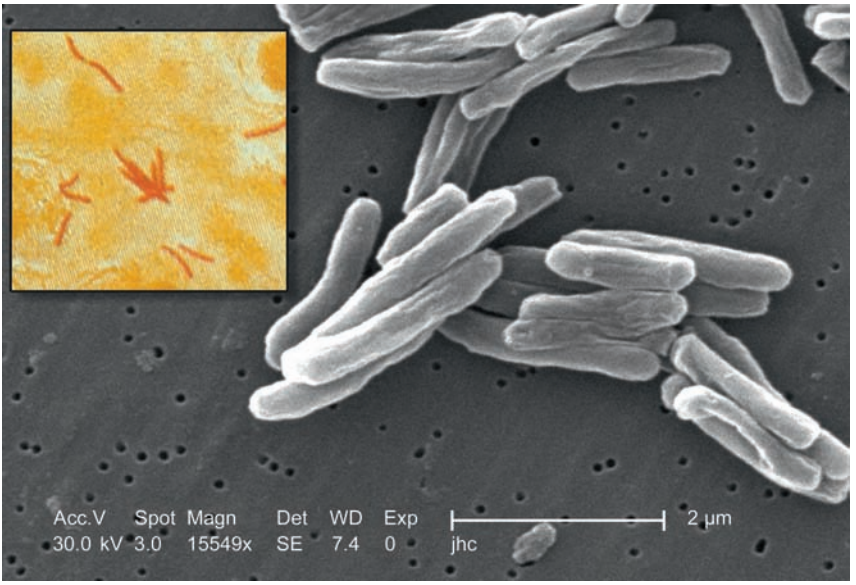

Figure 21.1 Scanning EM and light microscopy of *M. tuberculosis*. A corresponding light microscopy image (sputum) is depicted in the insert at the upper left (magnification unknown). Magnification of SEM approx. 15 500×. Both images are courtesy of the Public Health Image Library (http://phil.cdc.gov/).

compliance is a problem. Hence, slow-acting drugs in combination with poor health care systems – the vast majority of TB occurs in developing countries – lead to incomplete treatment and the emergence of drug-resistant MTB strains. Resistance usually occurs via mutations in the targets (or the activating enzymes) of the drugs [1].

Double resistance against the two first line drugs, isoniazid and rifampicin – termed multidrug-resistant (MDR) TB – is very difficult to cure and requires even longer therapy (18–24 months). More recently emerging extensively drug-resistant (XDR) TB (i.e., bacteria which are MDR and also resistant to the most potent second line drugs, fluoroquinolones and at least one injectable aminoglycoside) are becoming practically incurable [4–7].

Based on the two key issues, resistance and persistence, the objectives for TB drug discovery are clear. Most urgently, we need new drugs, with new mechanisms of action, that are at least comparable to current drugs in terms of potency, exposure, and tolerability, in order to keep drug-resistant TB at bay. As it turns out, this apparently simple task (simple, when compared with the problem of persistence) is not as straightforward as would have been expected at the beginning of the genomics and high-throughput biology era 10–15 years ago.

The failure to find new antibiotics is not specific to TB but is a general problem in antibacterial drug discovery. The paradigm of the 1990s, "genetic target identification → target-based high-throughput screen (HTS) → antibacterial lead compound

identified," does not work. Second and perhaps more importantly, we need to tackle the problem of persistence if we wish to control the TB epidemic. We need to develop new drugs that are drastically more efficacious in man. In contrast to the first issue, this is a disease-specific phenomenon. Are we using the right cell assay (growing culture) and the right animal model (mouse) to predict efficacy in man? The current "3M" approach "MIC/MBC → Mouse → Man → hope for the best" (MIC, minimal inhibitory concentration or concentration which inhibits growth; MBC, minimal bactericidal concentration or concentration which kills 99% of the culture) is unlikely to be the way to achieve the desired quantum leap, that is, a two-week cure for TB. Here we discuss the issues and the way forward around these two major objectives in TB drug discovery, finding "standard" antibacterial lead compounds, and tackling persistence. Considering the huge uncertainties we are facing, a sustained and systematic attack is required to understand and integrate TB biology, pathology, and pharmacology. Furthermore, it appears that we need critical appraisal of pre-existing compound collections held by pharmaceutical companies (pharma) and evaluate their usefulness for antibacterial drug discovery. A global, long-term effort is required in which industry, academia, clinical researchers, and funding agencies have to work closely together and bring their respective areas of expertise and capabilities to the table.

Drug-Resistant TB – Finding Lead Compounds with Cell Activity

In the standard antibacterial cell assay, a growing culture is exposed to a compound and it is determined at which concentration the compound inhibits growth (MIC). Next it is determined whether the compound has an additional cidal effect on the organism (MBC). If a compound only inhibits growth it is termed "static". If it kills the bacteria, it is "cidal". The best drugs we have for TB are cidal. Static drugs appear to be less efficacious. In order to improve the control and management of drug-resistant TB we need to discover new chemical entities (hitting new targets) that have an MIC as well as an MBC.

The Current Paradigm of Anti-Infective Drug Discovery

What is the present approach in the pharmaceutical industry to identify antibacterial compounds? Unexploited targets are selected on the basis of their genetic essentiality, epidemiology, assayability or HTS feasibility, and theoretical druggability, and preferably lack of human homologs. Inhibitors of those targets are obtained through HTSs of chemical libraries for hits. The hits are confirmed to be real (as opposed to promiscuous) inhibitors by biophysical, structural, and kinetic characterizations. Structure–activity relationships are developed and the hit series are profiled for physicochemical, *in vitro* pharmacokinetic and safety properties, and moved to the

growth-inhibition cell assay to determine whether the enzyme inhibitors actually show antibacterial activity [8].

The problem with the current approach to identify such lead compounds is that it does not work. So far, no antibacterial agent derived from the reductionistic "target-first" paradigm has reached the market. It appears that a serious bottleneck is encountered early in the process: the target-based approach does not deliver leads with attractive whole-cell activity, at least not with any reasonable efficiency. Payne and coworkers [9] described their experience with this approach to finding new antibacterial leads at GlaxoSmithKline (GSK), in which they evaluated 300 genes and carried out 70(!) HTSs. Most of the screens did not produce hits, and from those that did deliver a validated hit list, most hits did not show cell activity (note: this is an oversimplification made by the authors but appears to capture the essence of the GSK report). We reproduced the GSK experience for TB – much less extensively – over the past four years at NITD. For instance, screens against the shikimate kinase did not result in hits; screens against the pantothenate kinase resulted in attractive validated hit series with good potency on the target but with no or very poor growth inhibition activity in the cellular assay. In the case of peptide deformylase inhibitors, we identified compounds that showed whole-cell activity. However, *in vitro* growth inhibition kinetics were slow and resulted – perhaps not surprisingly – in rather poor activity in the TB mouse model, despite good tolerability and favorable pharmacokinetic properties of the compounds. It appears that "translating" activity against an enzyme into activity against a bacterium presents a major hurdle (meaning it does not work!).

Why It Does Not Work

The observation that target-based approaches for antibacterial drug discovery are not productive in delivering attractive leads with whole-cell activity has of course multiple reasons. Some of them are intrinsic to "technical details" of the process. Lack of druggability of the target might result in a poor hit list. The *in vitro* conditions of the biochemical assay might not mimic the state of the target inside the cell, resulting in false positives or false negatives. Some compounds might simply not penetrate into the cell, . . . and many more. These are general issues for target-based approaches in all disease areas.

However, the sheer dimension of the failure of target-based antibacterial drug discovery suggests that we are facing here two, more fundamental problems. The first problem concerns the usefulness of huge compound archives within the pharmaceutical industry for antibacterial drug discovery. Do we have the required "antibacterial" chemical diversity and properties to generate attractive hits? Second, even more importantly, there is very likely a problem in the predictive value of genetics, that is, the very concept underlying the current approach to finding antibacterial leads is flawed. An inhibitor against a protein encoded by a genetically essential gene might not necessarily result in (effective) growth inhibition of the bacterium. The inhibitor might not "copy" the "no growth" phenotype of the mutant.

The fact that a loss-of-function mutant of a particular gene does not form a colony on agar apparently does not (and not surprisingly so, at least in hindsight) predict that an inhibitor against the gene product will be an effective growth inhibitor. Why not? Should one not assume that inhibition of a pathway that delivers a product which is required for growth results in growth cessation? The question to be asked here is how "vulnerable" the pathway/target is to being shut down by a compound. To what level must the intracellular target be inhibited to block a pathway to a degree that causes growth cessation by 90%, 99%, 99.9%?

But worse: will inhibition of the target activate feedback loops that result in increased activity or expression of the pathway enzymes? Will a metabolic bypass be activated? Will inhibition of the target result in accumulation of a substrate that outcompetes a competitive inhibitor? We do not understand the kinetics, dynamics, and regulations of pathways sufficiently enough to predict whether an inhibitor will result in growth termination [10]. Only tool compounds, which we often do not have, can provide some answers to the above questions. Considering the lack of success obtaining growth-inhibiting compounds from genetically selected, target-based HTS projects, we might expect even less predictive value of loss-of-function mutants regarding the impact of an inhibitor on the viability of a bacterium. Knockouts of genetically essential genes do not form colonies on an agar plate. We can only speculate whether they are dead or alive.

What is the way forward after this apparent failure of the "genetic target identification → target-based HTS → lead compound identified" paradigm for antibacterial drug discovery? How can we identify novel leads that are growth inhibitory and cidal? In order to address this question one might look into the existing antibacterials that work and examine the drugs that are on the market to determine how they were discovered and what pathways they are targeting. Most importantly, we should understand how target modulation by a compound translates into cell death.

How Were Successful Antibiotics Discovered and Why Do They Work?

All existing antibiotics were discovered by phenotypic screens of natural product and synthetic compound libraries employing whole-cell screens. So the approach of placing antibacterial activity first for selection of hits is proven to deliver antibacterial drugs. Along the way, the targets for the – by then clinically validated – drugs were identified and employed to carry out further target-based improvements of the properties of the drugs. Walsh [11], Lange [12], and Silver [13] summarize the targets and mechanism of actions of current antibiotics. To name a few, the antibacterials that work in the clinic interfere with:

1. Peptidoglycan synthesis, via beta-lactams, glycopeptides (e.g., vancomycin);
2. Cell membrane integrity, via cationic peptides (e.g., daptomycin);
3. Protein synthesis, via aminoglycosides (e.g., streptomycin, kanamycin), macrolides (e.g., erythromycin), tetracyclines, and oxazolidinones (e.g., linezolid);

4. DNA replication and repair (e.g., fluoroquinolones);
5. RNA synthesis (e.g., rifampicin).

What we see here is a lot of "weird stuff" – weird from the perspective of how we currently select targets for our HTS campaigns and how we select hits for lead finding. We see for instance suicide substrates such as the beta-lactams resulting in covalent modification of their target (a standard no go for the pharmaceutical industry because of possible toxicity issues), we see substrate sequestering in the case of vancomycin, and we see membrane disruption (daptomycin). We see compounds (e.g., some ribosome modulators) interacting with several factors of multicomponent complexes, sometimes not even consisting of protein but RNA, and we see many compound classes interacting not with one but with multiple targets (e.g., beta-lactams, fluoroquinolones, daptomycin).

One striking impression is that none of the target–compound "couples" that work are of the category that would likely be selected or identified in our current HTS campaigns: where are the neat reversible, small molecular active site inhibitors of the simple, easy-to-assay, HTS-compatible enzymes? Are we simply wrong with the category or nature of the targets and hits we select, are we wrong with the target-related mechanisms of action we are chasing? We should question the starting principle according to which we follow only *one* target in the target-based approach, rather than multiple targets as demonstrated by dirty drugs in polypharmacology [13].

But let us move to the next question: how do cidal antibiotics kill? How is the chemical modulation of a target translated into its cellular consequence, death? Interestingly, despite a century of research, death of bacteria in response to antibiotic exposure remains a black box. Recently, Kohanski *et al.* [14] found intriguing evidence that events downstream of diverse antibiotics action converge on a cellular hub – the production of reactive oxygen species. The authors explored the impact of known bactericidal antibiotics by measuring production of hydroxyl radicals. They showed that cidal compounds targeting very different metabolic processes (fluoroquinolones/topoisomerase; aminoglycosides/ribosome; beta-lactams/peptidoglycan) all induce the formation of reactive oxygen species when tested in *E. coli* and *S. aureus*. In contrast, static antibiotics (e.g., chloramphenicol, tetracycline, erythromycin) did not induce production of hydroxyl radicals. In further elegant experiments, it was shown that formation of hydroxyl radicals occurs via oxidation of ferrous iron to ferric iron by peroxide and results in DNA and protein damage as the direct mechanism of death. Furthermore, a link to the TCA cycle and NADH level was established. This could indicate the existence of a unifying death pathway involved in the translation of target modulation by "cidal" compounds into the cellular death effect. Whether all this holds true in a more general sense remains to be determined. But it clearly shows that our knowledge of translating target modulation into bacterial death is rudimentary at best, even for the known antibiotics. What does that mean for the selection of novel targets – based on genetic and genomic data – if we are to identify cidal compounds? If we fail to understand how and why death occurs, it limits our ability to select targets that, when subjected to some kind (which one?) of modulation by a compound, are likely to induce death.

The Way Forward

Genetics and genomics are not predictive in selecting productive targets. We cannot deduce much from existing antibacterials, their targets, and their death mechanism, regarding the selection of novel targets that should lead to cell death. How can we identify novel, cidal antibacterial target/lead couples? The only pragmatic way forward is to place the high hurdle first and select for novel chemical entities that have antibacterial activity in cellular phenotypic screens, then use these compounds as tools to define the target and elucidate the mechanism of action (Figure 21.3a). If the tool compound has lead-like properties, one can move on and carry out cell-based lead optimization while target-finding activities are in progress. One can then test an optimized lead in animal models to demonstrate an effect of the compound *in vivo* and even progress to proof-of-concept studies in man to demonstrate a therapeutic effect. Once the target has been identified, target-based optimization can be carried out for the development of next generation drugs. If the tool compound did not possess lead-like properties and was therefore used as a tool only, it can constitute the starting point for target-based drug discovery programs. Essentially this means returning to the "compound that works first" approach applied during the "golden age" of antibiotic discovery and to forget the reductionistic "target first" approach. But this time we utilize all the power of modern genomics, genetics, molecular biology, and biochemistry to determine the mechanisms of action of the compounds and to identify targets faster than was possible in the past [15–17].

However, it is important to realize that following this "old/new" approach does not result in a quick fix. The normal attrition rate (100 projects result in one drug), the uncertainties, and the unpredictabilities intrinsic to drug discovery and development still apply. Returning to cell-based approaches removes *one* major hurdle: the hurdle of achieving cell activity. However, all other roadblocks and uncertainties – briefly achieving tolerability and favorable pharmacokinetics – remain [8]. Drug discovery is clearly a numbers game. But with phenotypic approaches, we get at least across the first major hurdle, achieving an effect on the pathogen. We start with compounds that have the desired effect at a relevant biological level.

Disease-Specific Challenges in TB Drug Discovery – Finding Lead Compounds Which Will Cure TB in Two Weeks

It takes 6–24 months of intensive combination therapy to cure TB and prevent relapse with antibiotics that have proven activity *in vitro*. In contrast, many pulmonary infectious diseases can be cured following monotherapy with similar drugs taken for only one to a few weeks. Why is long-term therapy required to cure TB [18]? To understand which properties of new TB drugs need to be improved in order to shorten chemotherapy, we need to understand why the

existing drugs are so ineffective. How the current first and second line drugs were discovered and optimized against tuberculosis can give us some insight into their limitations.

Para-amino-salicylic acid (PAS) and streptomycin (SM) are among the oldest drugs used to treat TB patients (see Figure 21.2 for the chemical structure of major antiTB drugs and current TB drug candidates in clinical development). They were discovered based on their *in vitro* activity against cultured TB bacilli. They were then tested simultaneously in the mouse and guinea pig experimental models of TB infection [19–21] and in patients [22, 23]. While SM is bactericidal at 2 μg/ml *in vitro*, PAS has little if any bactericidal activity against *M. tuberculosis* at clinically relevant concentrations. It resulted that therapy duration required to reach cure with these two drugs combined was around 18 months, and resistance to SM was quick to appear. Isoniazid (INH) was introduced a couple of years later and was tested in patients based on its much improved MIC compared to PAS and SM. However, it did not result in significant treatment shortening, possibly due to its bactericidal or MBC activity being similar to that of PAS and SM.

A somewhat notable shortening of antiTB therapy came with the discovery of rifampicin (RIF), which had a lower MIC against *M. tuberculosis* than SM and PAS [24], together with bactericidal activity at submicromolar concentrations. Yet, six to nine months of combination therapy were still required to obtain cure in patients with drug-sensitive TB. In contrast, RIF can cure recurrent/resistant staphylococcal infections within five days in combination with Novobiocin [25], or inhalation Anthrax within a few weeks in combination with a fluoroquinolone [26]. This massive discrepancy in therapy effectiveness, while *in vitro* potency against MTB and other pathogens remains similar, points to the unique pathophysiology of tuberculosis as the major reason for the long treatment duration.

All TB drugs were originally adopted based on their *in vitro* potency and activity in early models of TB infection in mice and guinea pigs. Today, the animal models have significantly evolved, but the MIC/MBC → Mouse → Man paradigm is still largely accepted for the optimization and clinical development of new TB drugs, such as the nitroimidazoles PA824 and OPC67 683, the ATP synthase inhibitor R207 910, and existing fluoroquinolones, to name a few. Could it be that the 3M approach introduces an intrinsic flaw in compound selection and that we reproducibly optimize for attributes that are not relevant to the real human TB disease? If this is the case, significant shortening of TB therapy is unlikely to be reached anytime soon.

What could be misleading, biased or missing in the 3M approach?

The Pathology of TB Disease in Animal Models Does Not Reflect that Found in Patients

Human necrotizing granulomata are not unique to tuberculosis and have a number of aetiologies [27, 28]. The histopathological manifestations of infection with *Mycobacterium tuberculosis* come about due to the interaction between bacterium and host. We recognize primary tuberculosis is more common in neonates [29], that there

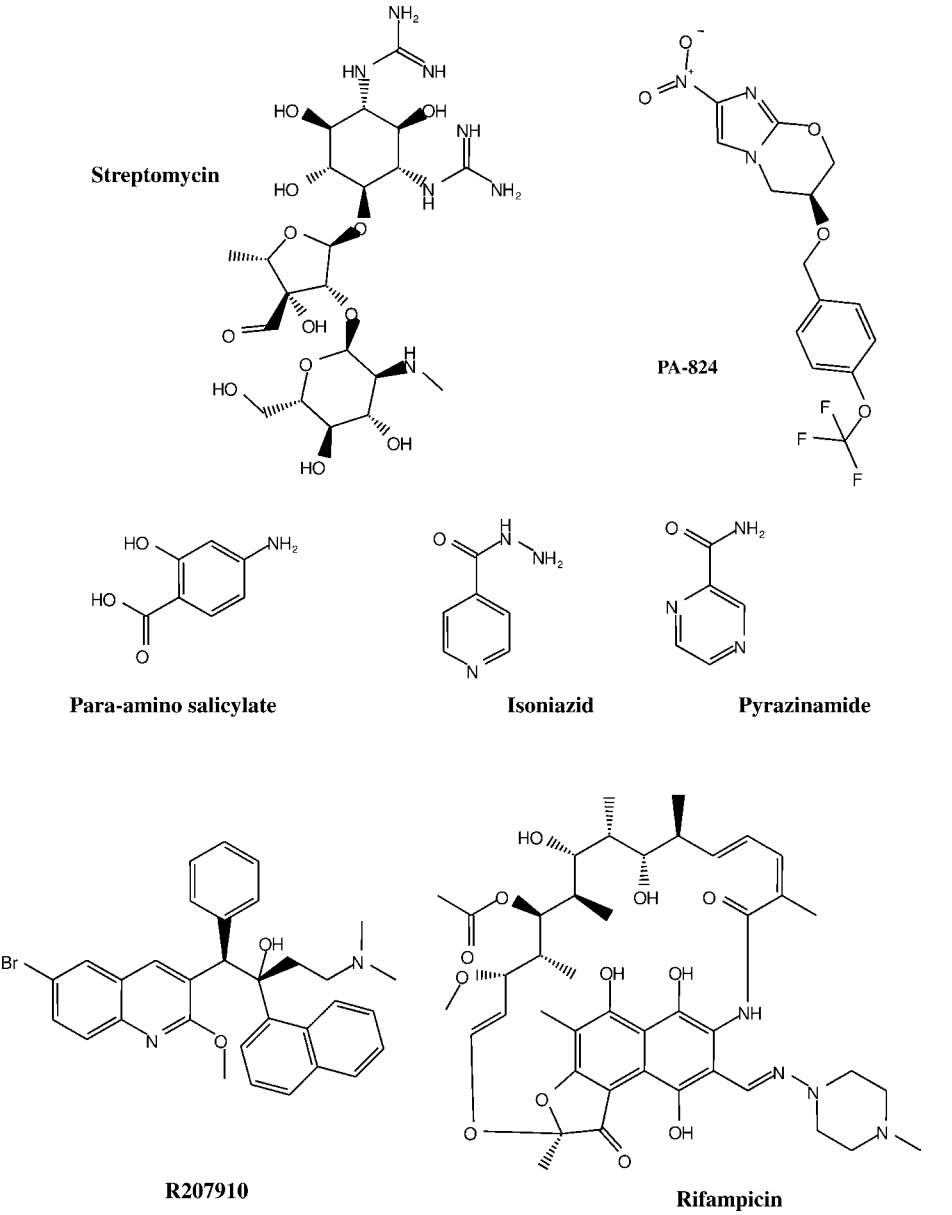

Figure 21.2 Chemical structures of antiTB drugs. Chemical structures of: (i) the major first line antiTB drugs: isoniazid, rifampicin, and pyrazinamide; (ii) some common second line drugs: para-amino-salicylate (PAS) and streptomycin; and (iii) two novel candidates currently in phase II clinical development: PA824 (Global Alliance for TB) and R207 910 (Johnson & Johnson).

is still much to be discovered about latent tuberculosis and why only 10% progress [30, 31] to post-primary reactivation that is the main reason for illness in adults [28]. The "granuloma" which medical students are shown in their first week of pathology teaching, and which diagnostic histopathologists recognize at a glance, is a structure which both protects the host from overwhelming infection but also makes it extremely difficult to eradicate using pharmacotherapy. The histological image is a snapshot in time. *In vivo*, the granuloma is a dynamic immune response which attempts (unsuccessfully) to contain the pathogen [31]. A good animal model used should therefore replicate the key elements of this environment (Figure 21.3c).

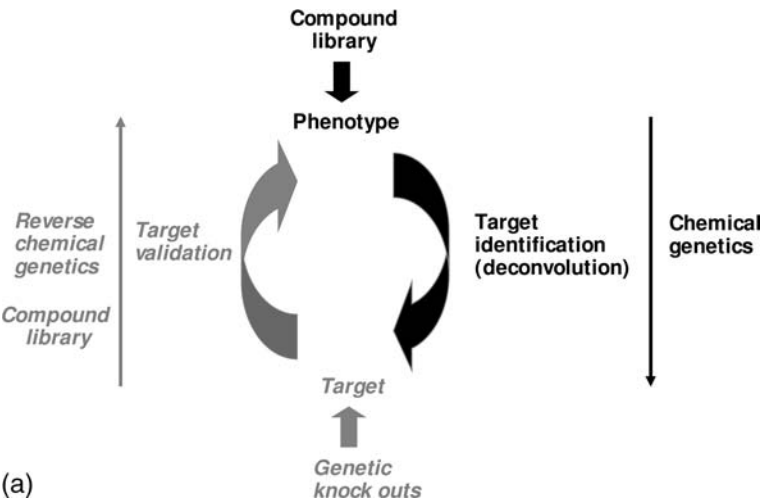

Figure 21.3 Key issues and the way forward in TB drug discovery. (a) New screening strategies: illustration of compound driven (black) versus genetics driven (gray italics) approaches for compound screening and target identification. In the classic genetics-driven approach, the starting point is a target identified via genetic knockout and later chemically validated via reverse chemical genetics. In the compound-driven approach proposed here, or chemical genetics, one places the higher hurdle first by screening for compounds inducing a desired phenotype in whole-cell assays, and later identifying the target to enable optimization of the compound class. (b) New predictive cell assays: one example of an optimized cell assay adapted to identify compounds specifically active against persistent cells which are suspected to be found in TB lesions. The growth curve of bacilli in sealed tubes is depicted with two cell density read-outs: optical density and CFU. *M. tuberculosis* self-depletes oxygen and becomes quiescent and phenotypically drug-resistant. The model is designed to mimic gradual depletion of available O_2 in two stages of microaerophilic (NRP-1) and anaerobic (NRP-2) persistence. Complete depletion of oxygen is indicated when cultures that are grown in the presence of the indicator methylene blue decolorize under anaerobic conditions. "d" indicates decolorization of the oxygen indicator methylene blue; CFU, colony forming units; A600, spectrophotometric absorption at 600 nm; NRP, nonreplicating persistence stage. (c) Proposed strategy to identify predictive animal models of TB infection. Multidimensional comparative study to characterize the various lesion types found in different animal models and in humans in terms of histology, bacteriology, host and pathogen gene expression profiling, pharmacokinetics, and local immune response. The aim is to determine which lesion types are most difficult to sterilize, identify which animal models exhibit such lesions, and understand the lesion-specific mechanisms underlying treatment failure.

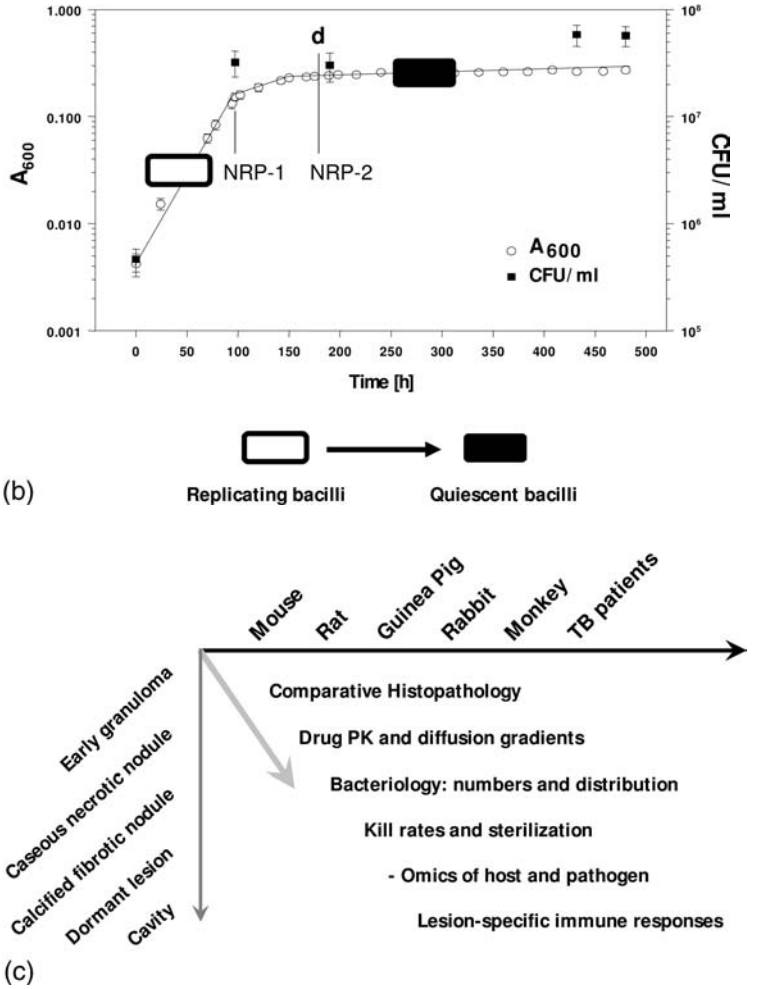

Figure 21.3 *(Continued)*

In the human, the histological appearances of tuberculosis are characterized by developed reactivated heterogenous lesions, larger than those found in most animals, in which necrotizing (caseating) necrosis and fibrosis are prominent histological features and secondary complications commonplace. The granulomata in TB tend to be rounded with pushing borders. Sometimes irregular, extensive "geographically shaped" necrosis can occur. The necrotic zones can contain amorphous granular debris or ghosts of alveolar septae ("infarct-like necrosis") [27]. Bronchiolar involvement is common although there are reports of airway-centered distribution similar to bronchocentric granulomatosis. The thin, beaded bacilli are visible with Ziehl–Neelsen acid-fast staining or auramine–rhodamine fluorescent staining and found within the debris of necrosis, though demonstrating this histologically can be

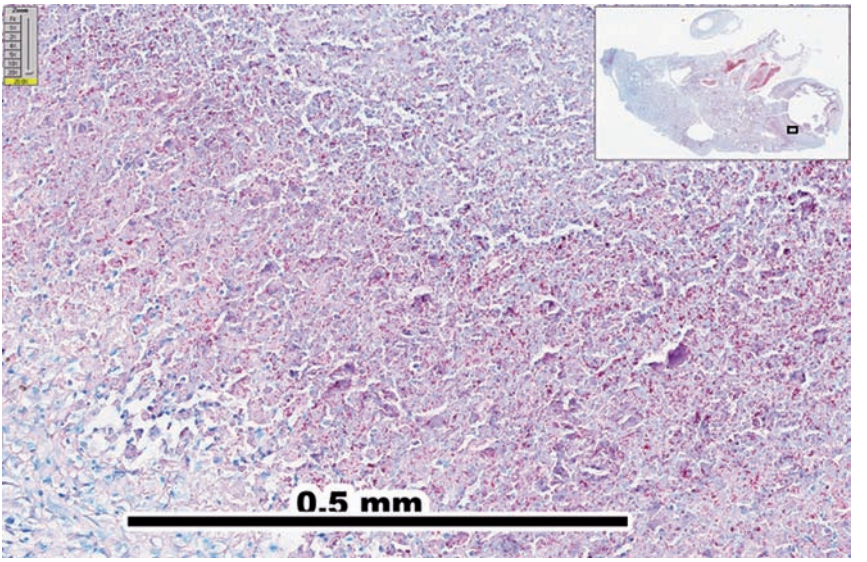

Figure 21.4 Acid-fast bacilli Ziehl–Neelsen (ZN) staining. Identifying unequivocal acid-fast bacilli on ZN staining is often difficult to visualize from human histology, as 100× oil immersion objective magnification is recommended. In contrast, this example of an induced infection in rabbit demonstrates abundant acid-fast bacilli in a necrotic area in lung (20× objective magnification). Scale bar shows 0.5 mm. (Outbred New Zealand white rabbit, 12 weeks post-infection with HN878, from Dr. Gilla Kaplan, Laboratory Mycobacterial Immunity and Pathogenesis, The Public Health Research Institute of New Jersey Medical School/ UMDNJ.)

difficult (Figure 21.4) and often underestimates the true number of bacilli present compared with detection by PCR [32].

In human TB it is common to see a mix of both necrotizing and non-necrotizing granulomata within a single microscope field. On purely morphological grounds, this is difficult to distinguish from the appearances of other fungal or mycobacterial infections. Numerous textbooks have discussed the differential diagnosis of granulomatous inflammation in the lung [28]. However, the purpose of this chapter is not to debate the diagnostic features or histological mimics, but to ascertain the differences between animal models and the human condition and how these may be reconciled.

Heterogeneity is demonstrated by the wide range of appearances of tuberculous granulomata (Figure 21.5) and some academic pathologists relish subclassifying them based on their appearance (despite the lack of evidence indicating this has any prognostic value). However, most diagnostic pathologists are more concerned with simply demonstrating acid-fast bacilli to confirm their diagnosis before moving onto the next billable case. There is no formal classification system. Histological variations include suppurative granuloma with prominent central neutrophil infiltrate, solid cellular granuloma without necrosis dominated by epithelioid histiocytes, solid sclerotic granuloma in which most of the lesion is composed of paucicellular

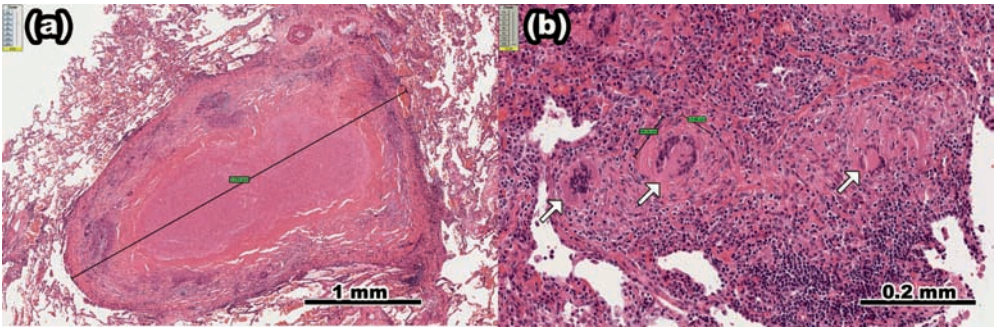

Figure 21.5 Human TB sections, standard haematoxylin and eosin (H&E) histochemical stain.This shows a solitary 3.3 mm tuberculous granuloma (a) in an adult human demonstrating the classic features of central caseating necrosis, peripheral epithelioid histiocytes with lymphoplasmacytic cells, scattered Langhan's type giant cells (b, arrows), and surrounding fibrosis. (Tissue blocks from Dr. Clif Barry III & Dr. Laura Via, Tuberculosis Research Section, LCID, NIAID, NIH, Bethesda.)

concentric sclerosis with a minimal chronic inflammatory cell infiltrate, and of course the calcified granuloma in which mineralization may have replaced all but the periphery of the lesion. There are certainly more variations. The diversity and heterogeneity of lesions can be attributed to the age of the lesion, variability in immunocompetency in the human host, and the fact that like any pathophysiological process in the body, an inflammatory reaction is not monomorphous – it has a range of dynamic histological manifestations.

Necrosis and fibrosis are distinctive features that are not always well represented in animal models, though we have seen it in examples from monkey (Figure 21.6), rabbit, and guinea pig. Absence of necrosis and fibrosis may be due to the significantly shorter time period – most animal models are primary infections. Sometimes what is seen in animal models is a granulomatous inflammatory reaction, rather than true granulomas (Figure 21.7). There is (imperfect) histological similarity between human and non-human infection. The issue is not whether the human infection looks like animal but rather whether the animal model can be made to look more like human within a practical time frame. It is self-evident that, at some early time point, human infection appears as a nonspecific aggregate of histiocytes, and this resembles many animal models; but histologically this does not fulfill the criteria for TB.

In looking towards improving animal models, we should first decide on what are the histological features of the human tuberculosis which have a genuine impact upon prognosis. Are there lesions subtypes which are easier to treat than others? Clinically, we recognize that the cavitating lesion which communicates with an airway is highly infective and difficult to treat and can have a post-surgical prognostic impact. This has led to speculation that the fibrotic outer wall of the granuloma in this instance is inhibiting the effectiveness of pharmacotherapy. Yet prominent normal vascularity can be demonstrated within many examples of such fibrosis (Figure 21.6). Necrosis grows proportionally more as lesion size grows, but unfortunately there is

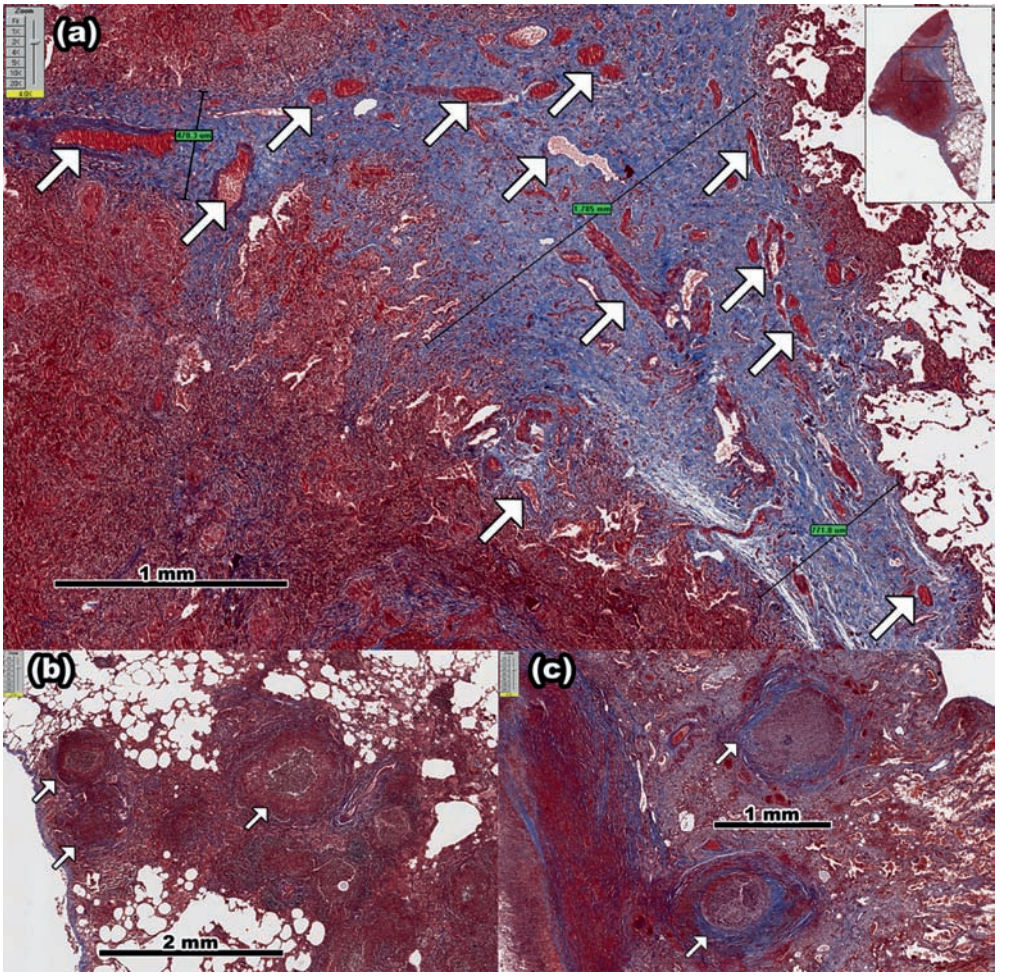

Figure 21.6 Fibrosis surrounding a granuloma. Image (a) taken of the edge of a tuberculoma in adult human demonstrates a thick fibrous wall up to 2 mm in thickness (Masson's trichrome stain, collagen in blue). Yet there are also prominent vessels present within this fibrous wall and these can be found extending all the way through and are well distributed (arrows). Image (b) shows well formed necrotizing granulomata (arrows) in monkey lung after more than 12 months post-infection. The scale bar shows 2 mm. The monkey is able to demonstrate the diversity of lesions seen in human, though in this example, the fibrotic changes are not as pronounced as seen with human tuberculomas (c, arrows) of equivalent size where encircling collagen bands are visible at low power. This may reflect the shorter time period (the age of the human lesions is unknown), differing immune responses, or limited sampling. (Cynomolgus macaques, *Macaca fasciularis*, tissue blocks from Dr. JoAnne Flynn, Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine.)

no data which proves that necrosis is a prognostic factor as it is in many neoplastic conditions. If even only a small percentage of necrotic cores act as repositories for *Mycobacterium*, then diffusion of drugs into this debris is an important parameter to be considered. Diagnostic histopathology literature recommends beginning the search for bacilli within the necrosis of the granuloma [27, 28] on the basis of diagnostic experience and past histopathological studies [33, 34]. However, other authors have used PCR to demonstrate bacilli on patients who died of unrelated causes, many of whom did not even demonstrate granulomata [35]. Ulrichs and Kaufmann believe that host–pathogen interactions actually occur *outside* the granuloma rather than at the interface between necrosis and cellular layer. They promote the idea that the majority of *Mycobacterium* are in the antigen-presenting cells in the periphery of the granuloma and that the necrotic core represents an "abandoned battlefield" with many necrotic areas "devoid of bacteria" [36]. The scope of this chapter precludes detailed discussion of other theories such as the possible role of the macrophage in shielding mycobacterium from antibiotics and the role of apoptosis in exposing these bacilli (and perhaps assisting treatment).

The reasons why tuberculosis treatment is suboptimal is multifactorial but if we were to focus on the histological phenotype of animal models then perhaps we should attempt to recreate both the size of the granuloma, the proportion of necrosis, and the presence of secondary complications. It would be incorrect to favor animal models that demonstrate far smaller lesions, a lack of heterogeneity, and little or no necrosis (Figure 21.7). We know that it is possible to create complexity in animal lesions (Figures 21.6, 21.7e) but if this is not emphasized, its significance will be forgotten among those who lack experience with human histopathology. Knowledge of a deficiency in an animal model does not preclude using that model, but unreserved defense of an animal model for historical reasons is short-sighted.

Even if we were to replicate the perfect tuberculous necrotizing granuloma, we should also remember that we are treating not the granuloma, but the entire lung. We should not be so focused on the tuberculoma that we fail to appreciate the context. In post-primary reactivation, which represents the majority of adult cases, radiologists report heterogeneous opacities in apical and posterior segments of upper lobes and superior segment of lower lobes, often associated with cavitation, especially in upper lobes. Parenchymal involvement is multisegmental with opacities that are sometimes ill-defined, sometimes with nodular and linear components, sometimes associated with distortion of adjacent bronchovascular and mediastinal structures. Radiologically, tuberculous granulomata are round or oval, sharply defined lesions (0.5–4.0 cm), solitary or multiple, sometimes with calcification. Secondary pathology such as tuberculous pneumonia, obstructive atelectasis, and overinflation from compression by adjacent enlarged nodes is common and pleural effusion is an occasional finding with or without tuberculous pneumonia.

This total pathophysiological condition is what contributes to the overall morbidity and mortality of this multiorgan lesion-based infectious disease. The scope of this chapter precludes a discussion of extrapulmonary tuberculosis or of the impact of intercurrent illness such as HIV/AIDS, but there is little doubt that these also compound the difficulties of eradication and recovery. In summary, the histopathology

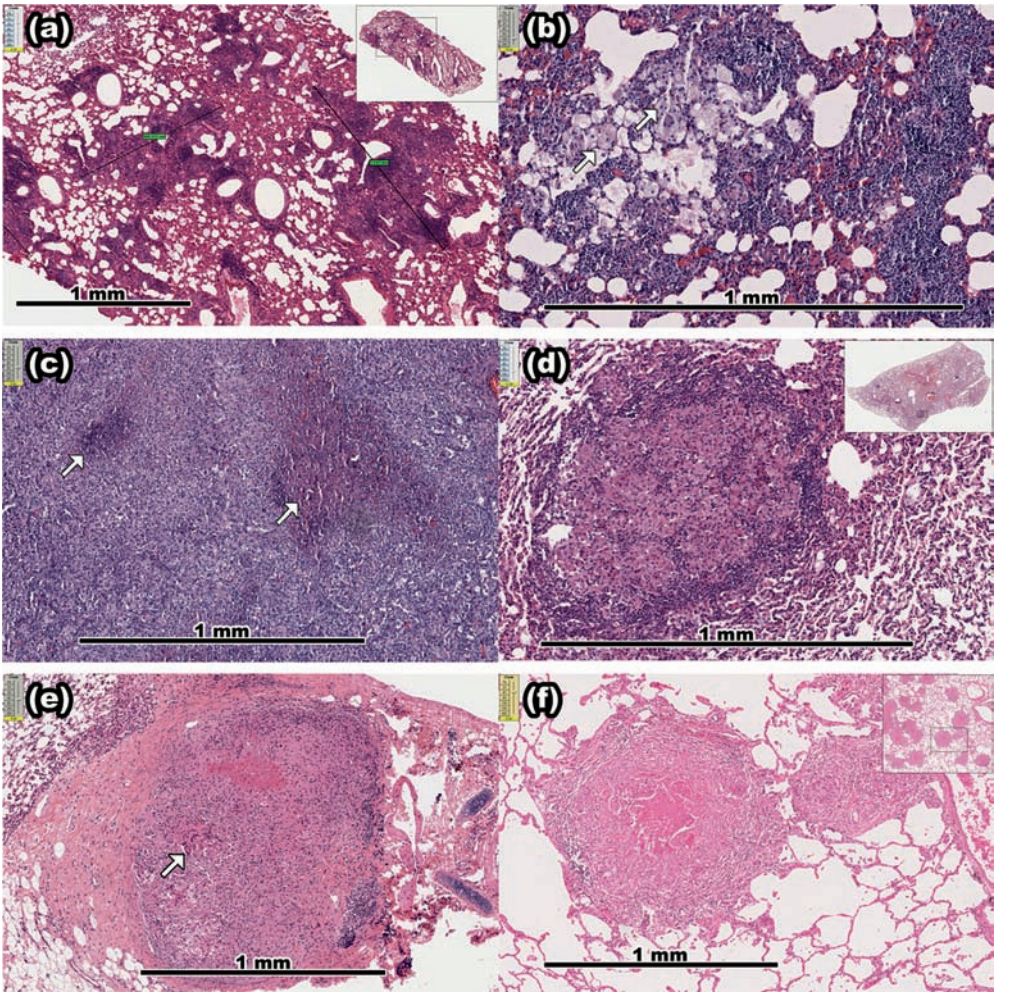

Figure 21.7 TB histology in different animals. The examples shown are by no means a definitive spectrum. (a) In this haematoxylin and eosin (H&E) stained section from mouse lung there is diffuse spreading granulomatous inflammation with prominent lymphoid aggregation without true granuloma formation. (Inbred BALB/c mice, eight weeks post-infection with H37Rv 27 294, from Dr. V. Balasubramanian and Dr. Sowmya Bharath, AstraZeneca India Pvt. Ltd., Bangalore). (b) An H&E-stained section of rat tissue demonstrates spreading chronic inflammatory cells and foamy histiocytes but no clear granulomas, no giant cells and no fibrosis. Scale bar shows 1 mm. (Wistar rat, six weeks post-infection with Strain W4, inoculum 10^6, from Dr. Pablo Bifani, Pasteur Institute, Molecular Pathology of Tuberculosis, Brussels). (c) This demonstrates a rampant tuberculous infection in rat lung with areas of necrosis (arrows). Scale bar shows 1 mm. (H&E staining, Lou Nude rats, 10 weeks post-infection with Strain W4, inoculum 10^6, from Dr. Pablo Bifani). (d) This isolated non-necrotizing granuloma in the rabbit would not be immediately recognized as TB if it were found in the human lung. The slightly collapsed but otherwise normal background lung is also notable. Scale bar shows 1 mm. (H&E staining. Outbred New Zealand white rabbit, eight weeks post-infection with CDC1551). (e) The section from guinea pig taken at 90 days post-infection (H37Rv) demonstrates a well

of MTB in the human is more than just a necrotizing granuloma, it is a complex spectrum of inflammation and repair, characterized by proportionally larger areas of necrosis and fibrosis than we see in animal models which results in an architecture which replaces and distorts normal lung. With larger lesions, the best outcome one could hope for is quiescent fibrosis – a return to normal lung histology does not seem possible. In truth, at this point in time we have more questions than answers – we can argue that many animal models are too simplistic histologically, but we cannot show evidence of what aspects of the complexity seen in humans are significant barriers to pharmacotherapy and therefore should be modeled.

Standard Cell-Based Assays Do Not Mimic the Biology and Physiology of TB Found *In Vivo*

One of the reasons for the poor efficacy of antiTB treatment is often referred to as "phenotypic drug resistance" where the bacilli adopt a physiological state that renders them resistant to therapy in a manner which is not genetically transmitted; in other words the "persisters" are not drug-resistant mutants. In TB, this observation is mostly explained by two phenomena, one driven by microenvironmental changes [37] and the other being purely stochastic [38]. In the first instance, it is postulated that bacteria adapt to environmental cues and enter a quiescent state that renders them less susceptible to antibiotics. This phenomenon of persistence is being extensively studied using an array of molecular and cellular technologies and has been largely covered in many reviews [1, 39, 40]. The stochastic phenotypic heterogeneity, in contrast, is defined as metastable variation in cellular parameters generated by epigenetic mechanisms [41]. In this case, stochastically driven diversity ensures that some cells in any given population will survive the antibiotic insult [38].

Beside these two well recognized and described mechanisms, namely environmental adaptation and stochastic variation, one should recall a third, very simple, explanation for the observed slow eradication of TB *in vivo*: the slow killing of growing MTB *in vitro*. Comparison of kill rates and efficacies of RIF against *Staphylococcus aureus* (fast growing) and *M. tuberculosis* (slow growing) bacteria *in vitro* reveals a somewhat intriguing trend. In culture, a 2-log kill is obtained against TB in five days (K. Pethe, unpublished data), while a 4-log kill is seen after three days against methicillin-resistant *Staphylococcus aureus* (MRSA) [25]. Not surprisingly, this observation is reflected in efficacy data of mouse infection experiments. While 28 days of RIF monotherapy is usually required to see a 2-log decrease in lung colony-forming units (CFU) against TB, RIF used against *S. aureus* in various infection models, including pneumonia, leads to cure or drastic CFU reduction upon administration of

circumscribed necrotizing granuloma (scale bar shows 0.5 mm) with a thick fibrotic wall and focal giant cells (arrow). (H&E staining. Guinea pig tissue from Dr. Randall Basaraba, Mycobacterial Research Laboratory, Colorado State University.) (f) A lesion from this case of human miliary tuberculosis (see low-power overview in the top right of the image) demonstrates surrounding fibrosis and central necrosis. (H&E, scale bar shows 1 mm).

a single dose or after a maximum of 24 h treatment [42, 43]. In the clinic, six months or more of combination therapy with three additional drugs are required to cure drug-sensitive TB. In contrast, RIF can cure recurrent/resistant staphylococcal infections within five days in combination with Novobiocin [25]. Obviously here, the site of infection, microenvironment, and bacterial physiology are markedly different between infections caused by TB and *S. aureus*, which might significantly influence drug access and drug action. However, one basic parameter that differentiates the two pathogen species is their rate of growth. Could it simply be that, somehow, the slow killing of *M. tuberculosis* is related to its slow growth?

Mycobacteria have designed their cell wall more like a fortress than any other known bacteria, and *M. tuberculosis* more so than faster-growing mycobacterial species. Among other attributes of the outer membrane structure of *M. tuberculosis*, the low surface density of pores and longer pore channels compared to other species and fast-growing mycobacteria are suspected to account for: (i) a slow growth rate due to restricted uptake of polar nutrients, and (ii) the limited efficiency of small hydrophilic drugs in TB chemotherapy [44]. More explicitly, tuberculosis has evolved more than one mechanism to limit growth rate while at the same time minimizing xenobiotic susceptibility. Supporting this line of thought, both the growth rate and antibiotic susceptibility of *M. bovis* BCG are increased upon heterologous expression of the MspA porin of fast-growing *M. smegmatis* [45, 46]. In other words, the uptake of solutes and chemicals through the MTB cell envelope could be much slower than in fast-growing mycobacteria. This would provide an explanation for the slow-growth phenotype and at the same time account for the observed slow kill of growing tubercle bacilli.

Even more interestingly, *M. tuberculosis* seems to have evolved a strategy to protect itself from mildly acidic conditions encountered within the macrophage phagosome, through the closing of its native OmpATb porin at low pH [47]. *M. tuberculosis* porins are thought to be key proteins for the uptake of small hydrophilic drugs, such as isoniazid, pyrazinamide, and ethambutol, three out of the four current first line TB drugs. One interesting implication of the pH-mediated closure of OmpATb is its likely negative effect on the penetration of these small hydrophilic drugs into lysosome-engulfed bacilli.

AntiTB Drugs Do Not Effectively Reach and Penetrate the Various Sites of Infection

Most drugs exert their effect not within the plasma compartment, where their levels are traditionally measured, but in defined target tissues where they must be distributed [48]. Target site concentrations may be substantially lower than plasma concentrations and may vary greatly even among drugs of the same class, with moxifloxacin showing much better lung penetration than levofloxacin, for example. Similarly, penetration of drugs into abscesses – somewhat similar in morphology to TB lesions with an outer collagen wall, inner layer of leukocytes, and central necrotic core – has been shown to vary significantly depending on the thickness of the abscess wall, stage of encapsulation, degree of fibrosis and vascularization, and viscosity of

the fluid [49]. This differential penetration might be even more acute in TB disease where lesion diversity in size, location, structure, and cellular/acellular content is remarkable. In other words, lesion-specific drug penetration and distribution may be limited by: (i) the uneven vasculature of the tubercles, (ii) the physical barrier generated by necrosis, multicellular layers, fibrosis, and calcification, and (iii) the protein binding and physicochemical properties of the drug itself. Whether lesion penetration by the various TB drugs is a limiting factor and what lesion types are the most difficult to penetrate are questions that remain unanswered so far. This aspect of the TB drug pharmacokinetics has been largely neglected, probably owing to the lack of adequate technology and the limited availability of human samples for *in situ* determination of drug levels in TB lesions.

The current first line antiTB drugs have been in use for many decades, yet their ability to penetrate lung tissue is poorly characterized. Scanty investigations date mainly from the 1950s to 1970s with isoniazid (INH) and rifampicin (RIF). A study from 1953, with radioactive INH administered to three patients, indicated that INH and/or its metabolites were present in various lesion types at concentrations close to those seen in blood [50]. In contrast, a large-scale Russian study of several hundred TB patients undergoing lung resection [51] measured INH levels in blood, healthy lung tissue, granulomata, cavities, and pulmonary lymph nodes, between two and five hours post-dosing. Here, the team found INH concentrations that were significantly lower in healthy and diseased tissue than in blood. In that same study, RIF was also found to distribute poorly in the various lesions and in lymph nodes compared to blood. A study in Hungary was performed on 18 patients undergoing thoracic surgery for either lung carcinoma, bronchiectasis, tuberculosis, or malignant lymphoma. Lung tissue levels ranged between 30 and 60% of serum levels [52]. Except for the radioactive INH study, the read-out was a biological assay that may have been influenced by several confounding factors inherent to the biological matrix and the bacterial strains used to quantify drug activity. Overall however, these studies strongly suggest that penetration into diseased tissue is both drug-specific and lesion-specific, in agreement with what is reported for nonTB drugs in lung tissue and abscess fluid [49, 53–55]. It is important to keep in mind that studies which measure drug concentrations in lung fluid or tissue homogenates have intrinsic limitations because they fail to differentiate between intracellular and extracellular levels, particularly when targeting intracellular pathogens. These limitations have a demonstrated clinical impact, because such values only reflect an average of vascular, interstitial, and intracellular drug concentrations, which invariably limit evaluation of site-specific phenomena and tend to neglect at least two potential barriers: macrophage uptake [56] and penetration into TB bacilli or "intracellular PK". This limitation was nicely illustrated in a recent study where no correlation was found between overall lung partitioning of fluoroquinolones and their efficacy in a classic mouse model of TB infection [57]. Thus, there is a clear need to repeat, refine, and expand drug penetration studies in TB lesions, by taking advantage of modern, innovative, and state of the art technologies.

Microdialysis is a well established method for the measurement of free antibiotic concentrations at the site of infection [58], and its value has been demonstrated to

describe time–concentration profiles of drugs in the interstitial space fluid of the lung [59]. Continuous monitoring of the bronchial epithelial lining fluid has been performed in anesthetized pigs, providing longitudinal information on the evolution of tissue concentration of analytes [60]. Such technology used in the context of TB disease holds tremendous potential to follow free drug concentrations in the different subcompartments of various lesion types and to visualize how different drugs effectively distribute from healthy lung tissue into successive lesion layers. Because a close correlation between the clinical outcome and PK/PD parameters was demonstrated in infectious diseases of the lung, microdialysis is increasingly recommended by regulatory authorities like the European EMEA and American FDA to measure target site concentrations and to predict effective doses in anti-infective therapy [59, 61]. In an elegant multimodality study, PET and microdialysis were recently combined to measure intracellular ^{18}F-ciprofloxacin by modeling intracellular drug levels based on total tissue and extracellular concentrations [62].

Imaging mass spectrometry (IMS) detects the mass of an analyte using matrix-assisted laser desorption/ionization (MALDI) MS, thus enabling the imaging of known and unknown analytes without requiring the development of specialized labels. Several compounds can be detected simultaneously with micron and even submicron spatial resolution in complex biological matrices [63–65], allowing the direct measurement of drug concentrations across, for example, different compartments of various granuloma types. A three-dimensional MS image or molecular weight-specific map of the sample can be produced at any desired molecular weight [66].

Combining pharmacokinetic data obtained by microdialysis, IMS and standard tissue level measurements, one could develop lesion-specific and drug-specific PK models of small molecule diffusion from blood into healthy tissue and lesion layers. One useful long-term objective is the development of predictive *in vitro* assays for the optimization of lesion penetration properties of small molecule drug candidates.

How Do We Develop and Validate More Predictive Cell Assays and Identify the Most Relevant Animal Model for Each Step in the Drug Discovery Process?

At present, we do not know enough about each of the three issues described above (i.e., the disconnect between animal and human TB pathology, the phenotypic drug resistance phenomenon, and the effectiveness of drug penetration into lesions) to determine whether and to what extent they contribute to the long treatment time required with our current drugs. To support the discovery and optimization of new and better drugs, a systematic and concerted approach should be adopted by the TB community to carry out comparative lesion characterization across animal models and in humans. Among many parameters which need to be better understood are:

(i) the cellular and subcellular localization of the bacilli, (ii) the microenvironmental conditions experienced in those compartments such as pH, oxygen tension, and carbon source availability, (iii) the physical and chemical barriers present in each lesion type, and (iv) the actual drug concentrations and permeability coefficients of the common drugs in the various lesion compartments. Next, this knowledge has to be translated into a panel of *in vitro* and *in vivo* assays and models that will support not only lead optimization campaigns but also target identification and validation. Cellular assays with good predictive value should be designed to optimize a new compound's ability to kill mycobacterial cells under metabolic conditions that are relevant to the human TB disease: exponential aerobic growth, anaerobic quiescence, nutrient starvation-induced quiescence, intracellular replication in macrophages, quiescence in phagosomal acidic pH environment, and so on. Improving the predictive value of animal models in turn requires that we first determine what histological aspects of the human form of TB have a genuine impact upon prognosis, and which lesion subtypes are rate limiting in treatment success. The final stage will be a systematic validation of optimized cellular assays and animal models using human clinical trial data with existing and novel TB drugs (Figure 21.3).

A convincing example of such a process can be found in the work of Larry Wayne and his colleagues, which led to the progressive elucidation of the role of hypoxia in TB drug resistance and to the development of a cellular assay which mimics the microaerophilic conditions experienced by some populations of bacilli in human TB lesions. Since the 1950s, it has been postulated that bacilli found in inflammatory and necrotic tissues within the human host have adapted to a low-oxygen microenvironment induced by granuloma formation [67, 68]. In response to this environmental change, it was shown that they are able to shift their metabolism away from oxygen-dependent to anaerobic pathways [69]. These early observations were recently confirmed in animal models through labeling studies using the oxygen-sensitive probe pimonidazole [70, 71]. Sever and Youmans [67] also suspected very early that low oxygen tension was linked to acquired resistance to antiTB therapy. To test the hypothesis that a nonreplicating persistent state can be induced through progressive oxygen deprivation of growing mycobacterial cultures, Wayne and colleagues developed an *in vitro* model of "settling" culture in which bacilli were allowed to sediment and stop replicating [72]. They further showed that these cells were synchronized and able to resume normal growth when exposed again to standard oxygen tension [73]. Differences in the molecular physiology, antigenic composition, and metabolism of these "submerged" cultures versus actively growing cells were established in a series of elegant studies. The model was later refined to mimic gradual depletion of available O_2 in two stages of microaerophilic (NRP-1) and anaerobic (NRP-2) persistence [74] and was designated the non-replicating persistence model (NRP) (Figure 21.3b). In the next logical step, it was shown that, during this *in vitro* anaerobic adaptation, the bacilli become phenotypically resistant to front line antituberculosis agents [74], while acquiring a unique susceptibility to metronidazole, a drug specifically used against anaerobes [75]. Today, the NRP model has been widely adopted as a means to screen drug candidates for their ability to kill nonreplicating persisters. Genetic and genomic studies have demon-

strated that adaptation to low-oxygen conditions *in vitro* involves a specific, coordinated, and dedicated global regulatory program. Comparison of this programmed response with that seen in bacilli extracted from human lesions will help evaluate the predictive value of the model and warrant its systematic use in drug discovery. Based on the activity of metronidazole in the NRP *in vitro* assay and in the rabbit model of TB infection, a clinical study sponsored by NIAID was initiated in South Korea (http://clinicaltrials.gov/ct2/show/NCT00425113?term=metronidazole+Masan&rank=1), where the efficacy of adjunctive metronidazole to second line agents is evaluated in multidrug-resistant patients, using both established and exploratory clinical read-outs such as PET imaging of FDG uptake in the lungs (Clifton Barry, personal communication). If conclusive, the trial will reinforce the value of Wayne's NRP model and pave the way of a retro-validation approach from clinical data back to animal models and cell-based *in vitro* assays.

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22
Decreasing the Number of Gaps in the Draft Assembly of the *Mannheimia haemolytica* M7/2 Genome Sequence

*Hon Q. Tran, Mathias Beig, Volker Spehr, Andreas Rohwer, Gottfried Unden, and Paul M. Selzer**

Abstract

Numerous bacterial genome sequencing projects remain in draft status. The main reason for this is the time-intensive and expensive procedure which is necessary to close genome gaps. With increasing numbers of sequenced bacterial genomes, information from sequenced genomes of closely related bacterial strains can be exploited to decrease the number of gaps or even to finish the genome assembly. Therefore, we developed a gap-closure workflow which combines *in silico* genome comparison analyses with a molecular biology-based method. The procedure was applied to the draft genome sequence assembly of *Mannheimia haemolytica* M7/2, the major causative agent of severe pneumonia (also known as shipping fever) in cattle. The *in silico* approach utilized the genome sequences of the related strains *M. haemolytica* PHL213 and *Actinobacillus pleuropneumoniae* serovar 1 strain 4074 as templates for the gap closure in *M. haemolytica* M7/2. For the *in vitro* approach we used a specific technique for PCR amplification and contig extension. By applying this workflow we reduced the number of contigs in the *M. haemolytica* M7/2 draft genome sequence from 175 down to 14. Thereby the full-length sequence of 147 additional genes was realized and 24 novel genes were identified. Application of the draft assembly and gap closure approach achieved a 99.8% complete assembly of the *M. haemolytica* M7/2 whole genome sequence.

Bacteria of the HAP Group Cause Severe Infections in Animals

Bacteria of the *Pasteurellaceae* family belong to the γ-Proteobacteria and consist of the genera ***H****aemophilus*, ***A****ctinobacillus*, and ***P****asteurella*, known as the HAP group. This family notably harbors a number of animal pathogens: *H. somnus*, whose virulent form is an opportunistic bovine pathogen causing multiple wasting diseases

including pneumonia, septicemia, and abortion [1]; *A. pleuropneumoniae*, which is an economically important porcine lung pathogen that causes highly contagious pleuropneumonia [2]; and *P. multocida*, which is primarily the etiological agent of fowl cholera, but is a multispecies pathogen causing many serious diseases of significant economic importance, including respiratory problems in cattle [3]. However, the most important HAP pathogen causing pneumonia in cattle is *Mannheimia haemolytica* serotype A1 (formerly known as *P. haemolytica* [4]), a gram-negative, facultatively anaerobic bacterium. It is the primary agent of the enzootic bronchopneumonia [5], a respiratory disease also known as shipping fever in cattle causing an economic loss of more than US$ 10^9/year in North America [6, 7].

Life Sciences have been Revolutionized by Whole-Genome Sequencing Projects

In 1995, a remarkable milestone in the life sciences was reached with the release of the first completely sequenced bacterial genome – *H. influenzae*, a HAP-group organism – by the Institute for Genome Research [8]. For the first time, biologists could analyze a complete bacterial genome, including genes and their regulatory regions. Moreover, the availability of complete sequenced genomes enabled new scientific research approaches like comparative genomics, mutation screening, and metagenomics [9].

As of June 2008, over 650 microbial genome sequences are publicly available and more than 1000 genomes are currently sequenced, from all branches of the bacterial superkingdom (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). The number of bacterial genome sequences is expected to grow even faster as next-generation technologies revolutionize the sequencing market. Novel sequencers are able to process millions of sequence reads in parallel, thus massively reducing costs and increasing the throughput [10]. For example, while a microbial sequencing project in 1998 required over US$ 10^6 and more than one year for completion, ten years later such a project costs US$ 10 000–20 000 and can be finished within a few weeks. The high-throughput sequencing itself, however, is done within one day [11]. Therefore it is likely that, within the next years, most of the important pathogenic microorganisms of man, animals, and plants will be sequenced almost to completion [12], given the current coordinated MetaHIT (www.metahit.eu) and Gut Microbiome (www.ccfa.org) efforts.

All sequencing technologies produce reads which are usually between 25 and 1000 nucleotides in length. These reads are assembled into contigs and finally result in a completed contiguous bacterial genome sequence. However, despite the technical advances in genome sequencing, even a high-quality sequencing experiment does not immediately result in a completely sequenced genome independently of the technology used. Very often the assembly process leads to a draft genome sequence of individual contigs which are not connected to each other. The gaps between these contigs represent missing and therefore unknown nucleotide sequences. The closure

of the gaps is often based on time consuming PCR-based molecular biology techniques [13, 14], direct sequencing [15], representational difference analysis, for example, subtractive hybridization [16], or high-resolution macro-restriction mapping [17].

Sequencing the *M. haemolytica* Genome

Due to the importance of the *M. haemolytica* pathogen in animal health, Intervet started a drug development program by sequencing the genome of *M. haemolytica* M7/2, an A1 serotype isolated from cattle suffering with enzootic bronchial pneumonia. A whole-genome shotgun sequencing strategy (9.5× coverage) based on classic dideoxy chain termination sequencing technology [18] yielded 38 357 reads. These were assembled to a draft genome sequence consisting of 175 contigs of 1–80 kb in length (average 14 kb), with a total length of 2.588 Mb, and 2607 identified and annotated genes, including relevant antibiotic targets and virulence factors. The total genome size – analyzed by pulsed-field gel electrophoresis – was estimated to be about 2.8 Mb, from which we calculated that approximately 10% of the genome and about 250 putative target genes have not been sequenced (unpublished data).

Therefore, we established a gap closure workflow combining *in silico* and *in vitro* technologies with a subsequent gene identification approach in order to reduce the number of gaps and increase the amount of potential drug target genes from the *M. haemolytica* M7/2 draft genome sequence. The bioinformatic approach exploits the availability of two genome sequences from closely related bacteria. For the molecular biology approach we pursued two PCR-based strategies to gain new sequence information.

Comparative Genomics Enables Gap Closure

Gaining additional sequence information without DNA sequencing is a smart technique to close gaps within a draft genome sequence. This can be achieved by a comparative *in silico* approach using genomic sequences of related organisms as templates. For *M. haemolytica* M7/2, two genome sequences from closely related bacteria were available – the draft versions of *M. haemolytica* PHL213 [19] and *A. pleuropneumonia* serovar 1, strain 4074 (available at NCBI Microbial Genomes Resources: entries NZ_AASA00 000 000 and NZ_AACK00 000 000, respectively).

M. haemolytica PHL213 is of serotype A1 as strain M7/2 and was isolated from an infected calf in the United States. Its recently published draft genome sequence consists of 152 contigs ranging from 1 to 100 kb, leading to 2.569 Mb nucleotides in total [19]. Both *M. haemolytica* contig genomes share highly identical regions. Some *M. haemolytica* M7/2 contigs match over their whole length to *M. haemolytica* PHL213

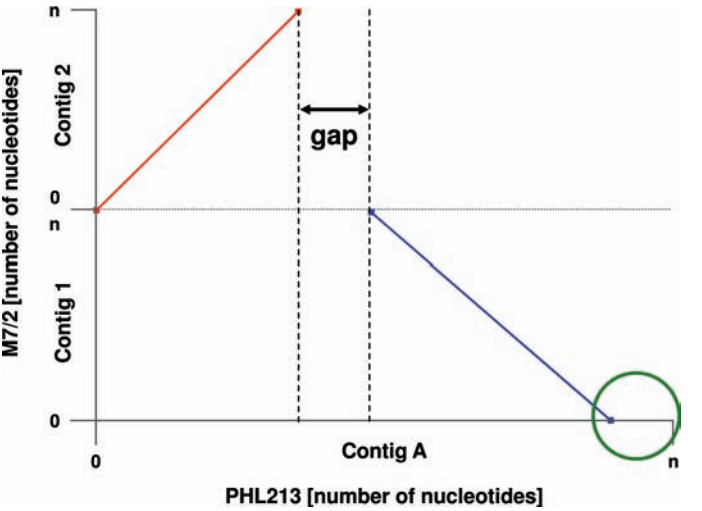

Figure 22.1 MUMmer dot plot of an *in silico* gap closure. Two *M. haemolytica* M7/2 contigs (*y*-axis) match to one PHL213 contig (*x*-axis) having a missing region (gap) in between. A second missing region of M7/2 contig 1 is indicated by the green circle. Red line: same orientation of the contigs, blue line: reverse orientation. This comparative genomics approach enabled the *in silico* closure of 99 gaps.

contigs. Other M7/2 contigs overlap with their counterparts in the PHL213 genome. The identity rate in these matching regions ranges over 80–100%, with an average of 99.5%. All matching regions between both *M. haemolytica* contig genomes are 2.54 Mbp in length, that is, 98% of the known *M. haemolytica* M7/2 genome sequence (2.588 Mb) could also be found in the genome sequence of the other isolate. Therefore, using the nucleotide sequences of *M. haemolytica* PHL213 for direct complementation of the incomplete genome of *M. haemolytica* M7/2 should be a feasible approach. We aligned both *M. haemolytica* draft genome sequences to each other in order to identify genomic regions which are available within the genome of PHL213, but absent from the M7/2 genome (Figure 22.1). For this comparison we used MUMmer [20], an open-source modular software package which rapidly aligns very large DNA sequences. Finally, the comparison between both *M. haemolytica* strains led to the *in silico* closure of 99 gaps at positions where homologous chromosomal arrangements were found in the M7/2 genome (Table 22.1) and the extension of 29 contigs.

Gap Closure Through Syntenic Relations

The second bacterial genome sequence we used to perform *in silico* gap closure was from *A. pleuropneumonia* serovar 1 strain 4074. *A. pleuropneumoniae* is a very close

Table 22.1 Combined *in silico* and *in vitro* gap closure.

Methods	Gaps closed	Number of *M. haemolytica* M7/2 contigs
		175
In silico		
Contig comparison between MH M7/2 and MH PHL213	99	76
In silico/in vitro		
Synteny-based approach using APP genome sequence	12	64
In vitro		
SPACE	7	57
Multiplex PCR	43	14
	Newly identified genes 24 Completed full-length genes 147	

relative to *M. haemolytica* and in phylogenetic analysis both form a lineage distinct from other *Pasteurellaceae* members [19], indicating that the degree of synteny between both genomes is very high. Based on this assumption, we compared the terminal genes of each of the 76 remaining contigs (152 proteins) of the *M. haemolytica* M7/2 genome sequence towards the *A. pleuropneumoniae* contig genome sequence. Thereby, we identified 23 gene/protein pairs of which each showed significant identity to neighboring genes/proteins of the *A. pleuropneumoniae* genome (Figure 22.2). Such a colocalization in *A. pleuropneumoniae* suggests that for each of the 23 gene/protein pairs the two corresponding M7/2 contigs are neighboring regions on the *M. haemolytica* genome. To test this hypothesis we generated 23 primer pairs in order to perform respective PCR reactions on the *M. haemolytica* M7/2 genomic DNA template. Twelve of the gaps could be filled (Table 22.1), which proved that synteny-based gap closure is a useful strategy, albeit limited by similarity of available homologous genomes.

PCR-Based Strategies for Gap Closure

Following *in silico* gap closure, we applied two PCR-based methods which aimed to connect the remaining 64 contigs by DNA amplification and sequencing. First, we developed a **s**pecific **P**CR-**a**ssisted **c**ontig **e**xtension (SPACE) which is a modification of methods described elsewhere [13, 21, 22]. We used AgeI (ACCGGT) which cuts the *M. haemolytica* genomic DNA, having a GC-content of 41%, statistically every 6506

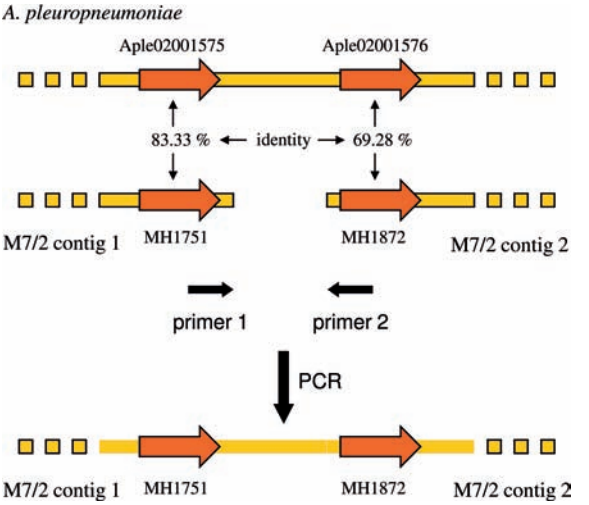

Figure 22.2 Synteny-directed gap closure. Synteny-based gap closure in *M. haemolytica* M7/2. The ORFs at all contig ends were compared against the *A. pleuropneumoniae* serovar 1 strain 4074 genome sequence. This example shows two *M. haemolytica* ORFs (MH1751 and MH1872) residing on different contigs which significantly matched to two *A. pleuropneumoniae* genes (Aple02 001 575 and Aple02 001 576) which are co-located. Due to the close relationship between both HAP bacteria it is assumed that M7/2 contig 1 and M7/2 contig 2 can be connected. To test this hypothesis we generated PCR primers for this gene pair bridging the genome gap and amplified the region using *M. haemolytica* genomic DNA as template. An additional 12 gaps were closed using this approach.

nucleotides. The method is based on the principle that the resulting DNA fragments will contain parts of the three following components (Figure 22.3a):

(i) DNA belonging to the contig, where the amplification starts;
(ii) DNA of the neighboring contig to be identified as such;
(iii) and DNA of the gap that connects both contigs.

In order to enable the PCR amplification, two opposite directed oligonucleotide primers were selected for the starting contig. Both SPACE primers should be unique and oligonucleotides showing high similarities toward more than one contig of the *M. haemolytica* M7/2 genome sequence were discarded.

After AgeI digestion, the fragments were religated using T4 DNA ligase under conditions that favor the formation of monomeric circles [23] (Figure 22.3b). Although the DNA fragments can still ligate to other fragments, substantial recirculation events occur that are sufficient to suit as template molecules for the subsequent SPACE PCR. In this PCR reaction the ligation products are amplified using primer 1 and 2 (Figure 22.3c), purified by preparative gel electrophoresis, and finally analyzed by direct PCR product sequencing. The newly determined DNA sequence was then aligned with contigs of the *M. haemolytica* M7/2 genome. In the case that parts unambiguously match with a second contig besides the starting contig, the nucleotide sequence of the gap was successfully identified and the two contigs could be

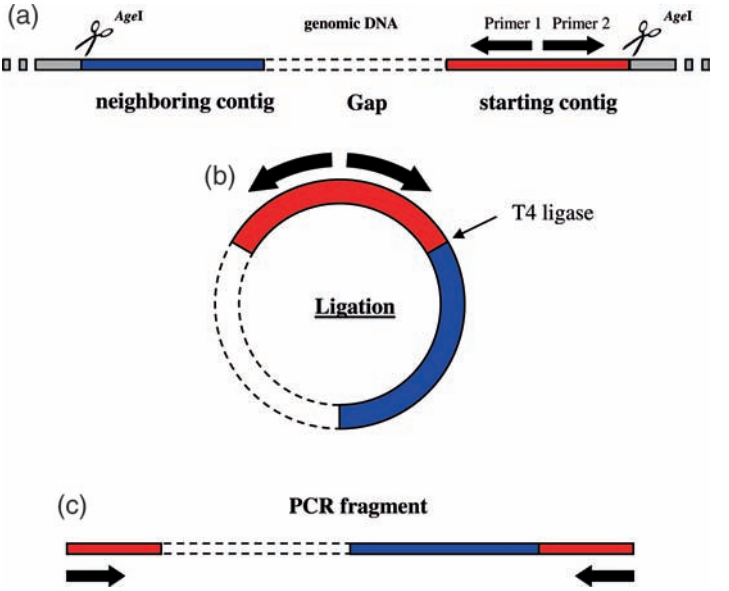

Figure 22.3 Specific PCR-assisted contig extension. (a) Genomic DNA is randomly cut with selected restriction enzymes. (b) After re-ligation monomeric circles are generated containing parts of the starting contig as well as of an unidentified neighboring contig and the nucleotide sequence of the bridging gap. (c) Gap closure was successful if the unknown contig flanking the gap can be uniquely assigned to a second *M. haemolytica* M7/2 contig, in addition to the starting contig. This approach enabled the closure of 7 gaps.

connected. Altogether, we applied SPACE to 29 starting contigs of which seven could be connected to other contigs.

Subsequently, we employed optimized Multiplex PCR as described [14, 24], a technology which was already used for gap closure in whole-genome shotgun sequencing projects. This methodology enabled the closure of an additional 43 gaps (Table 22.1).

The Newly Merged *M. haemolytica* M7/2 Contig Genome Contains Novel Genes

By applying our combined bioinformatics and molecular biology based workflow to the draft genome sequence of *M. haemolytica* M7/2 we could reduce the number of contigs from the original 175 down to 14 by closing 161 genome gaps (Table 22.1). In total, 74 154 bp of new DNA sequence were realized. In order to identify novel genes located within the newly determined genomic regions we annotated the new M7/2 contigs and compared the results with the annotation of the former contig genome

version. Finally, we identified 24 yet unknown genes and determined the full length of 147 genes, 16 of which have been assigned a new potential function. Thus, the aim of our study to close gaps in the draft genome sequence of *M. haemolytica* M7/2 in order to find novel potential target proteins was successful.

Conclusion

The advent of massively parallel sequencing technologies led to the increased availability of bacterial genome sequences. This enormous pool of information provides an opportunity to close gaps in yet incomplete bacterial sequencing projects and to finish genome assemblies using the information from genomes of related bacteria. We

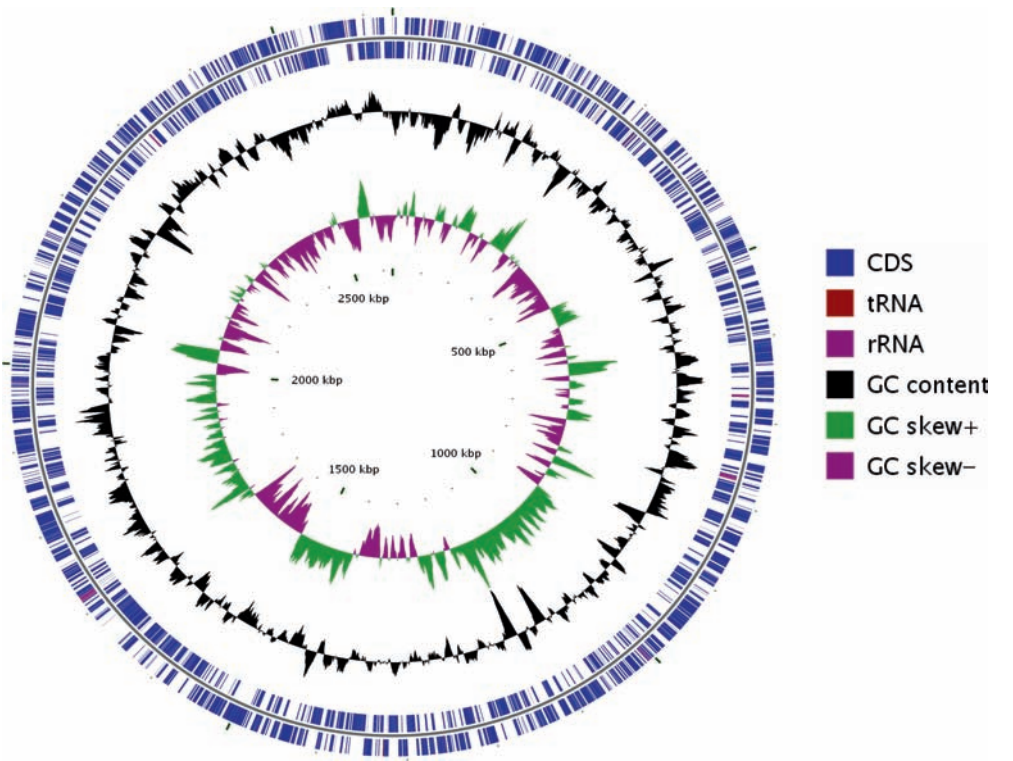

Figure 22.4 *M. haemolytica* M7/2 genome map. Circular representation of the *M. haemolytica* M7/2 draft genome sequence. The 14 contigs are arbitrarily ordered and each gap is filled with 317 Ns. Outer circle: CDS, coding sequence, tRNA, transfer RNA, rRNA, ribosomal RNA; middle circle: GC content, guanine-cytosine content; inner circle: GC skew+ and skew–, calculated using the equation $(G - C)/(G + C)$, G is the number of guanines and C is the number of cytosines. The genome map was created with the software CGView [25].

demonstrated the successful implementation of such a strategy and generated a workflow for gap closure in bacterial genomes. By combining *in silico* genome comparisons with molecular biology based *in vitro* methods, we effectively applied the workflow to the genome sequence of *M. haemolytica* M7/2:

(i) exploiting the information of a parallel genome project from another *M. haemolytica* isolate of the same species and serotype;
(ii) using the genome sequence of *A. pleuropneumoniae*, a closely related relevant of *M. haemolytica* with conserved synteny;
(iii) and applying PCR-based techniques for contig extension.

These approaches enabled the closure of 99, 12, and 50 gaps, respectively, which led to a reduction in the contig number from 175 to 14. The average length of the closed gaps was 317 bp. Assuming that the remaining gaps have approximately the same length, only 4.4 kb are missing of the complete *M. haemolytica* M7/2 genome sequence. Therefore, the estimated genome size decreased from 2.810 Mb as determined by pulsed field gel electrophoresis to 2.669 Mb. Taking this into account, the so far known 2.665 Mb represent 99.8% of the *M. haemolytica* M7/2 genome sequence. A circular representation of the genome is shown in Figure 22.4, in which the remaining 14 contigs are arbitrarily ordered and each gap is filled with 317 Ns.

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23
Total Synthesis and Configurational Assignment of Pasteurestin A and B, a Natural Product with Antimicrobial Activity on *Pasteurellaceae*[**]

Marion Kögl, Lothar Brecker, Ralf Warrass, and Johann Mulzer[*]

Abstract

In the pre-genomic era, natural products were the most important source for the discovery of new antimicrobial drugs. But even today, in the post-genomic era, natural products still are an inspiring and fruitful source for new chemical entities for the fight against live-threatening bacterial infections. A recent Japanese patent reported the isolation and biological properties of two novel basidiomycete sesquiterpenoids, pasteurestin A and pasteurestin B. Both compounds were obtained by fermentation of *Agrocybe aegeritta* and exhibited strong and selective antibacterial activities against some *Mannheimia haemolytica* strains, a pathogen causative for bovine respiratory disease (BRD). Here we describe the total synthesis and characterization of pasteurestin A and B. The determination for their antimicrobial activity of a large and diverse set on gram-positive and gram-negative bacteria revealed specific activity on some *Pasteurella multocida* while leaving other bacteria undisturbed.

Introduction

A Brief Synopsis of Antibiotic History

The discovery of the first synthetic antibiotic prontosil (**1**, Figure 23.1) in 1935 and penicillin in 1929 were major milestones of medicine [1]. War demands then led to the industrial production of penicillin G (**2**, Figure 23.1) in 1943. Other β-lactams were commonly used, such as cephalosporin and its derivatives or chloramphenicol (**3**, Figure 23.1).

[**] This chapter is dedicated to the memory of our colleague and friend Marion Kögl, who unexpectedly passed away on 26 August 2008 in Cambridge, UK.

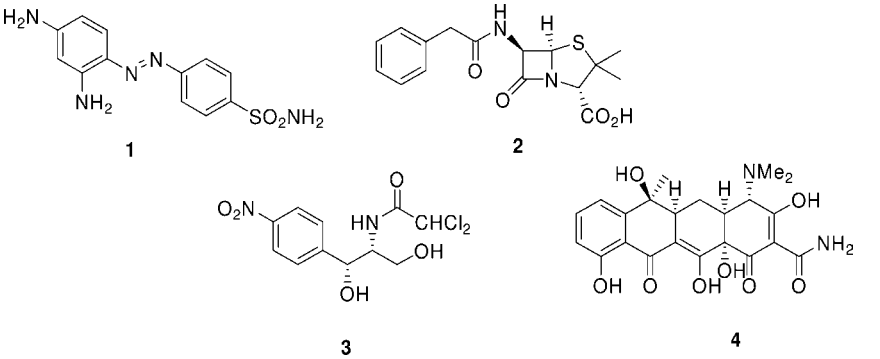


Figure 23.1 Prontosil (**1**), penicillin G (**2**), chloramphenicol (**3**), and tetracycline (**4**).

Other classes of naturally occurring antibiotics were introduced, among others tetracycline (**4**, Figure 23.1) and its derivatives, macrolides such as erythromycin A, and rifamycins, with rifampicin (**5**, Figure 23.2) as a prominent member of this class. Also worth mentioning are vancomycin and the aminoglycosides. Additionally, a few purely synthetic antibiotics such as the quinolones and fluoroquinolones like ciprofloxacine (**6**, Figure 23.2) or oxazolidinones (**7**) found widespread applications. Despite the discovery of these compounds, bacterial infections are still one of the most important threats for human and animal health. The situation seems to be even more critical due to the global increased resistance development of pathogens towards the most common and recently introduced antibiotics. The very limited number of market entries and the drying pipeline for new antibiotics is the result of

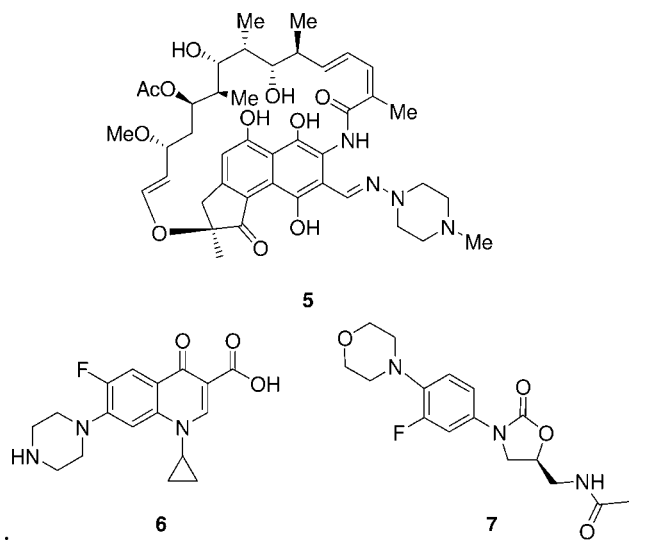


Figure 23.2 Rifampicin (**5**), ciprofloxacin (**6**), and linezolid (**7**).

the major players in the pharmaceutical industry losing interest in antibacterial research. The discovery of new leads and chemical entities for the enforcement of the antibacterial arsenal is nevertheless desperately needed.

New Lead Structures

Antibiotics are a group of pharmaceuticals which did not profit from the introduction of genomic and high-throughput screening (HTS) methods [2] due to their multifaceted mode of action. In fact, most antimicrobial lead compounds in the past were found first by *in vitro* tests and the determination of the minimum inhibitory concentration (MIC) on various bacterial cultures. Often, the mode of action of the molecules was determined at a later stage. New lead structures from natural sources can be identified by combining state-of-the-art biochemical methodology and molecular modeling techniques, which allows identification of the natural products' binding motives. In order to obtain first information on structure activity relationship and compound properties, the laborious total chemical synthesis of natural products and their synthetic and semisynthetic derivatives has often no alternative. It represents the only way to obtain compounds in sufficient quantity for *in vitro* and *in vivo* evaluation. The screening of natural sources still remains one of the most attractive ways to find new drug targets. A key to access bacterial vulnerability is certainly the use of natural products as scaffolds for antibiotics.

Disease-Focused Treatment

In order to reduce the use of broad spectrum antibiotics and to minimize the risk of resistance development, it is proposed to use antibiotics that exhibit their antibacterial activity only on illness-associated pathogens. One example might be the treatment of "shipping disease", a serious bacterial lung infection in cattle (also known as bovine respiratory disease, BRD) with heavy economic losses in the United States. The pathogenic bacterial species that are associated with this respiratory infection include mainly *P. multocida* and *M. haemolytica*, two pathogens from the *Pasteurellaceae* family. It is believed that the treatment of this disease with *Pasteurellaceae*-specific antibiotics with no activity on enteric bacteria (e.g., *E. coli*) reduces the risk of resistance development. Antibiotics used today in the veterinary field are all products of human antibiotic research programs with rather broad-spectrum activities. Examples are tulathromycin, danofloxacin, cefquinome, marbofloxacin, and florfenicol. Since *Pasteurellaceae*-specific antibiotics do not have an application in human health, the finding of these compounds has to rely on dedicated animal health research programs.

In the following we describe one such effort. The *Pasteurella*-specific natural products pasteurestin A and B were found in a Japanese patent. Total synthesis of these biologically active protoilludane derivatives provided the natural compounds for configurational assignment and MIC testing.

Total Synthesis as a Basis for Configurational Assignment of Natural Products and Access to New Derivatives

Pasteurestin A and B

In 1992, a mushroom of the strain *Agrocybe cylindracea* Maire K-3793 (*A. aegeritta*) was discovered. As a result of its cultivation, two novel sesquiterpenoid antibiotics named pasteurestin B (**8**) and A (**9**) were isolated and patented in 2002 [3]. Both compounds exhibited strong and selective activity against some *Mannheimia haemolytica* strains, a pathogen causative for BRD [4]. The physical and chemical characteristics determined did not allow the determination of the compounds' relative or absolute configuration, only the connectivity of their carbon skeleton was ensured.

The structure of both pasteurestins is characterized by an unusual angular and strained hydrocyclobutaindane nucleus and an α,β-unsaturated β-hydroxy moiety on the six-membered ring. Pasteurestin B (**8**) contains five contiguous stereocenters, pasteurestin A (**9**) four contiguous and one isolated quarternary stereogenic center. For both compounds, the relative configurations at C-4a, C-7a, and C-7b were assumed to be the same as in other protoilludanes, represented by illudol (**10**, Figure 23.3) [5–7]. The stereocenters at C-4 and C-6 in **9** and C-4 and C-7 in **8**, respectively, and the absolute configurations had to be established by total synthesis.

This was the only way to prove the proposed structures on the basis of ^{1}H and ^{13}C NMR data, IR spectra, masses, and comparison to other basidiomycete sesquiterpenoids. The absolute configuration can be assigned by a comparison of the specific rotations of the natural and the synthetic compound. Second, a variable total synthesis allows further biological tests and can serve as an excellent basis to get access to different derivatives for later SAR studies.

The Protoilludane Family and their Biological Relation

The pasteurestins belong to the family of protoilludanes, which in turn belong to the family of basidiomycete sesquiterpenoids that exhibit considerable toxic, antibiotic,

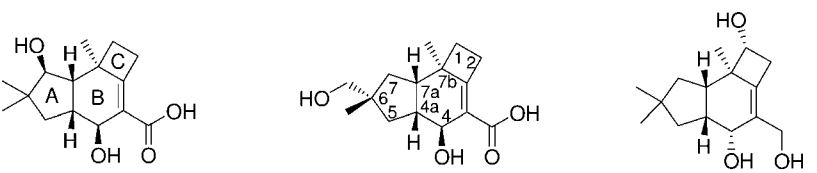
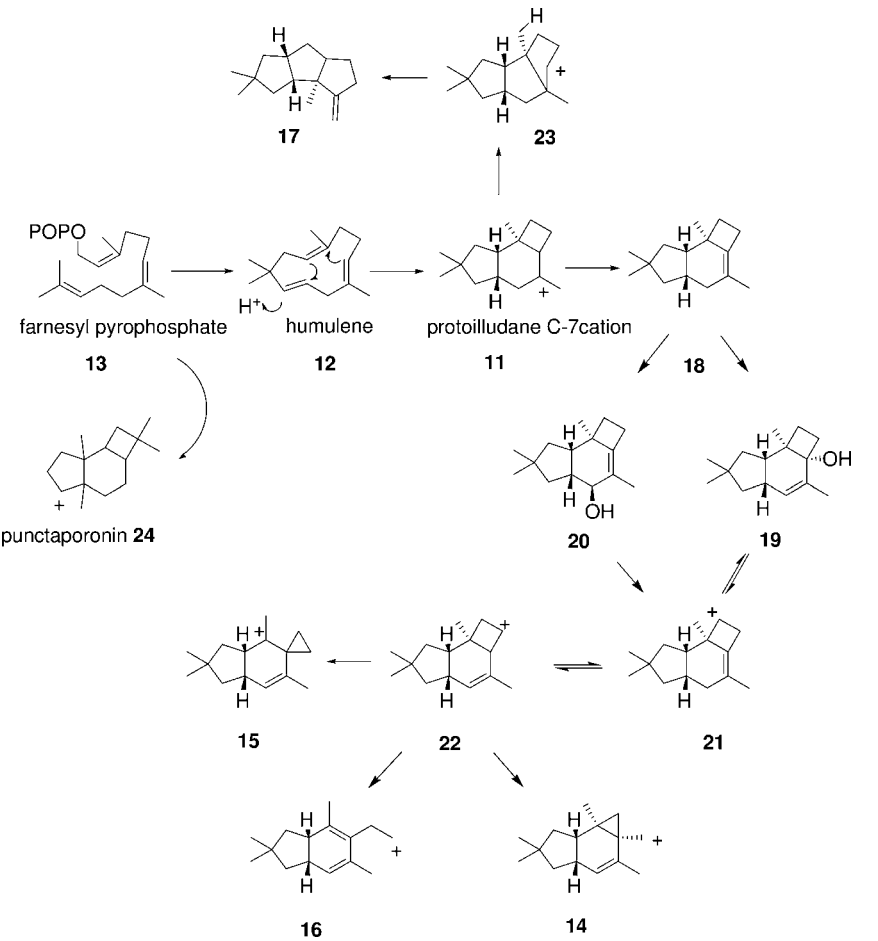


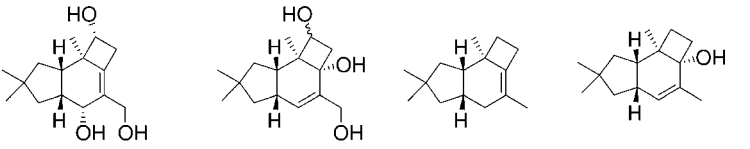
Figure 23.3 Formerly assumed configuration of pasteurestin B (**8**), pasteurestin A (**9**), and structurally related protoilludane illudol (**10**).

and antitumor activity [8]. Basidiomycetes are a particular class of highly specialized and advanced fungi, their name deriving from "basidium", the cell in which meiosis occurs [9]. Sesquiterpenes are the major constituents of the secondary metabolites produced by these fungi. They are usually produced as chemical defense agents against predators. An overview of the structural diversity of the illudoid family, their natural occurrence, biosynthesis, and activity is given here.

Many compounds from diverse members of the subclass *Basidiomycotina* have been isolated and characterized. Some relationships between the illodoids are shown in Scheme 23.1 [10]. Protoilludanes arise from a biogenetic cationic cyclization of humulene (**12**), which is the cyclization product of farnesyl pyrophosphate (**13**). Compound **18**, which may be formed from the protoilludane C-7 cation (**11**) by deprotonation, might be oxidized to the corresponding allylic alcohol (**19**) or its equivalent (**20**), which would rearrange to a cyclobutyl cation (**21**) with the



Scheme 23.1 Hypothetic relationship between members of the illodoid family.



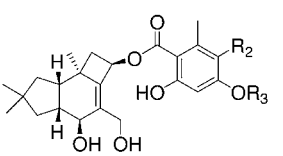
10 **25** **26** **19**

Figure 23.4 Illudol (**10**), neoilludol (**25**), Δ^6-protoilludene (**26**) and Δ^7-protoilluden-6-ol (**19**).

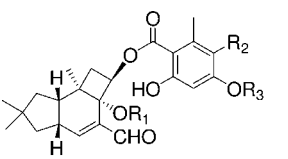
protoilludane skeleton. Through the carbonium cation (**22**), illudane (**15**), illudalane (**16**), and marasmane (**14**) type carbon skeletons may be formed. The protoilludane C-7 cation (**11**) can also be directly converted to the hirsutane skeleton (**17**). Protoilludane materials have been isolated from all cultures which yield these sesquiterpenes. Fungal metabolites containing the protoilludane skeleton are the most numerous among fungal sesquiterpenoids.

Illudol (**10**, Figure 23.4) is a metabolite from the basidiomycete *Clitocybe illudens* (e.g., *Omphalotus olearis*), a poisonous mushroom, commonly named "Jack o' Lantern" because of its bioluminescent property [11, 12]. Neoilludol **25** has also been isolated from *C. illudens* [13] (*O. olearis*), while a possible precursor, Δ^6-protoilludene (**26**), as well as Δ^7-protoilluden-6-ol (**19**) were isolated from *Fomitopsis insularis* [14]. These three compounds were, together with illudol, the first members of the huge class of protoilludanes to be isolated.

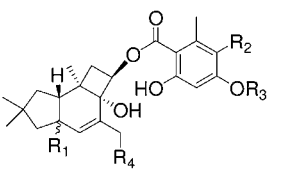
The family of illodoids isolated from *Armillaria mellea* (**27–41**, Figure 23.5) includes several compounds with antibiotic activity [15–18]. Preparations of *A. mellea* are used medicinally in China. In 1997, five additional derivatives of melliolide (**41**) were isolated from *A. tabescens* grown in culture [19]. Unlike those of most mush-

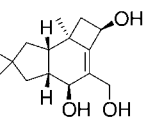


27 R_1= OH, R_2= H, R_3= H armillyl orsellinate
28 R_1= O, R_2= H, R_3= H
29 R_1= OH, R_2= H, R_3= CH_3 armillyl everninate
30 R_1= OH, R_2= Cl, R_3= CH_3 arnamiol
31 R_2= OH, R_2= OH, R_3= H judeol



32 R_1= H, R_2= H, R_3= H melleolide
33 R_1= CH_3, R_2= H, R_3= H methyl melleolide
34 R_1= H, R_2= H, R_3= CH_3 armillarin
35 R_1= OH, R_2= Cl, R_3= CH_3 armillaridin

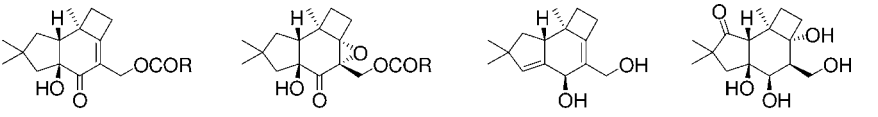




41 armillol

36 R_1= Hb, R_2= H, R_3= CH_3, R_4= CH_2OH melleolide B
37 R_1= OHb , R_2= H, R_3= H, R_4= CH_2OH melleolide C
38 R_1= OHb R_2= Cl, R_3= CH_3, R_4= CH_2OH melleolide D
39 R_1= Hb R_2= H, R_3= H, R_4= CH_2OH melledonol
40 R_1= Hb R_2= H, R_3= H, R_4= CHO melledonal

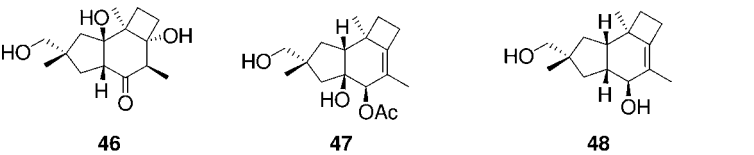
Figure 23.5 Illodoids from *Armillaria mellea*.



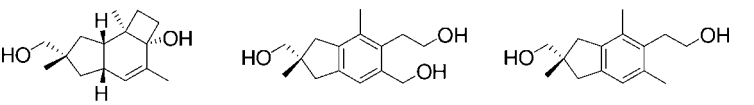
42 **43** **44** **45**

R = $C_{17}H_{33}$ (oleic; 90 %) and C_{17} H_{31} (linoleic, 10 %)

Figure 23.6 Structures of atlanticones A–D (**42–45**).



46 **47** **48**



49 **50** **51**

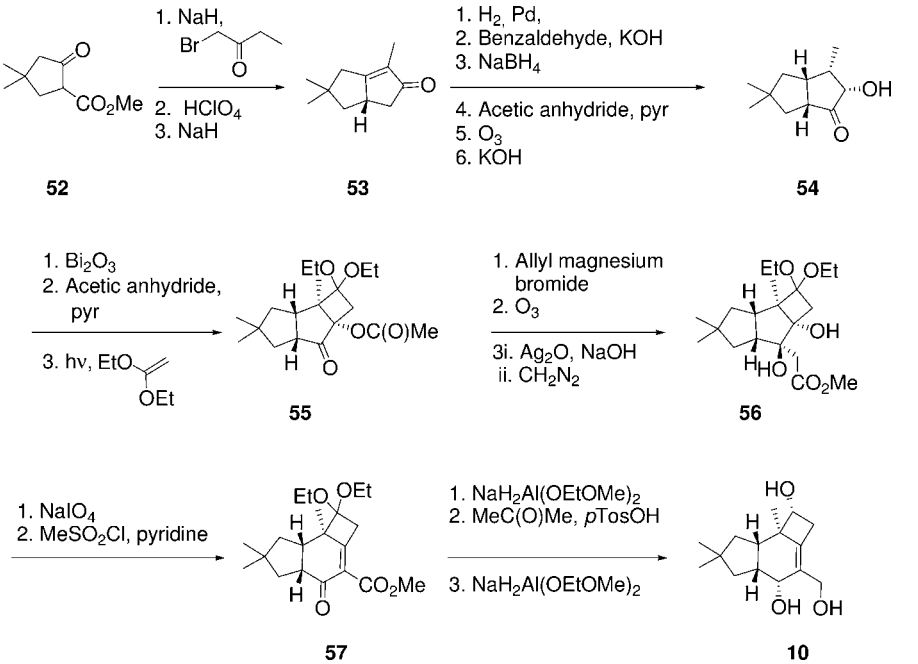
Figure 23.7 Structures of russujaponols A–F (**46–51**).

rooms, the fruiting bodies of *Lactarius atlanticus* are rarely attacked by worms, insects, and other parasites. Some other organisms display similar deterrence, often associated with the presence of secondary metabolites constituting a chemical defense system. In the *Russula* and *Lactarius* species, these defense mechanisms are generally not present in intact fruit bodies, but are formed enzymatically from inactive precursors once the mushroom is injured. The "inactive forms" are sometimes long-chain fatty acid esters of different sesquiterpene alcohols which are, upon injury, exposed to different lipases or esterases. As a result, the free alcohols are released and converted enzymatically into rearranged activities, usually endowed with potent biological activities, included antibiotic and cytotoxic ones [20]. Also from *Lactarius atlanticus*, different compounds were isolated, namely atlaticones A (**42**) and B (**43**) from intact carpophores whereas atlanticones C (**44**) and D (**45**) were isolated from injured fruiting bodies (Figure 23.6).

The last group of illodoid sesquiterpenes to be presented are the russujaponols A–F (**46–51**), which were isolated in 2006 from the fruiting bodies of *Russula japonica* Hongo (Figure 23.7) [21].

Total Syntheses of Illudol

Illudol (**10**) has been synthesized three times in racemic form, the first synthesis was completed by Matsumoto *et al.* (Scheme 23.2). Catalytic hydrogenation leads to the *cis*- annelation of the later hydrindane moiety which in turn induces the

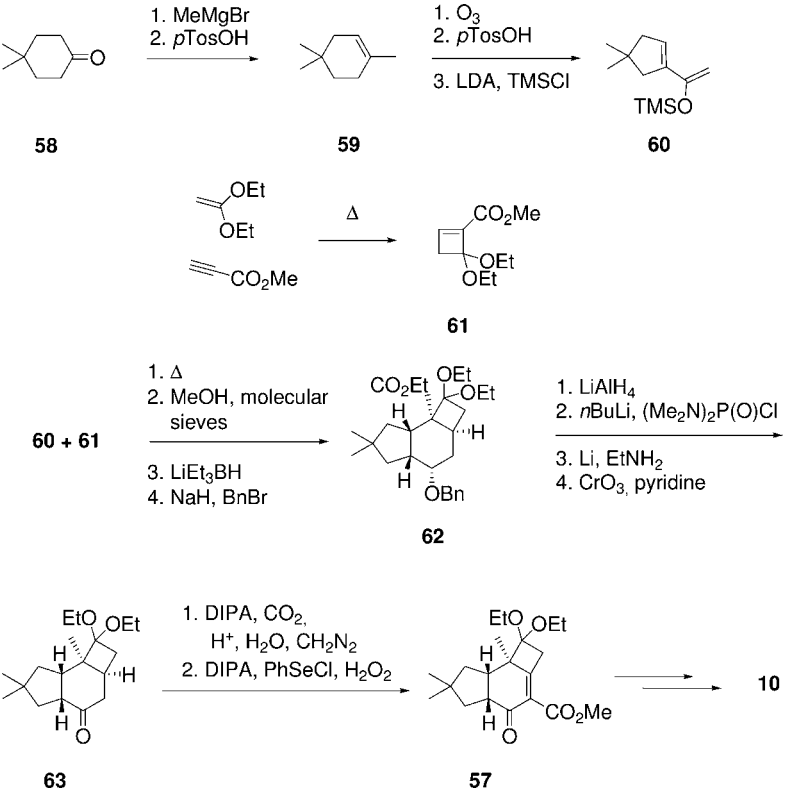


Scheme 23.2 Matsumoto's total synthesis of illudol (**10**).

anti-configurational outcome of the cycloaddition. The six-membered ring is formed by ring expansion.

After alkylation of ketoester **52** with 1-bromobutan-2-one, the resulting diketoester was decarboxylated to afford a diketone. This was subsequently subjected to an intramolecular aldol condensation, resulting in α,β-unsaturated ketone **53**. After hydrogenation, an aldol condensation with benzaldehyde delivered the kinetic α-phenylmethylidene ketone which was subsequently reduced and acetylated. Ozonolysis followed by saponification of the acetate resulted in α-hydroxy ketone **54**. This material was oxidized affording the α,β-unsaturated ketone, acetylated, and subsequent photochemically induced [2 + 2] cycloaddition provided stereospecifically tricyclic **55**. After treatment with allyl magnesium bromide and ozonolyzis, the resulting hydroxyl aldehyde was finally oxidized to the acid. Under these conditions also the ester was hydrolyzed and the material was esterified to give **56**. Glycol cleavage of the five-membered ring was followed by aldol reaction to close the six-membered ring and the remaining mesylated hydroxyl group was eliminated. After final reduction of both ester and ketone moiety in compound **57**, the acetal was liberated and the carbonyl was also reduced to afford illudol **10** (Scheme 23.3).

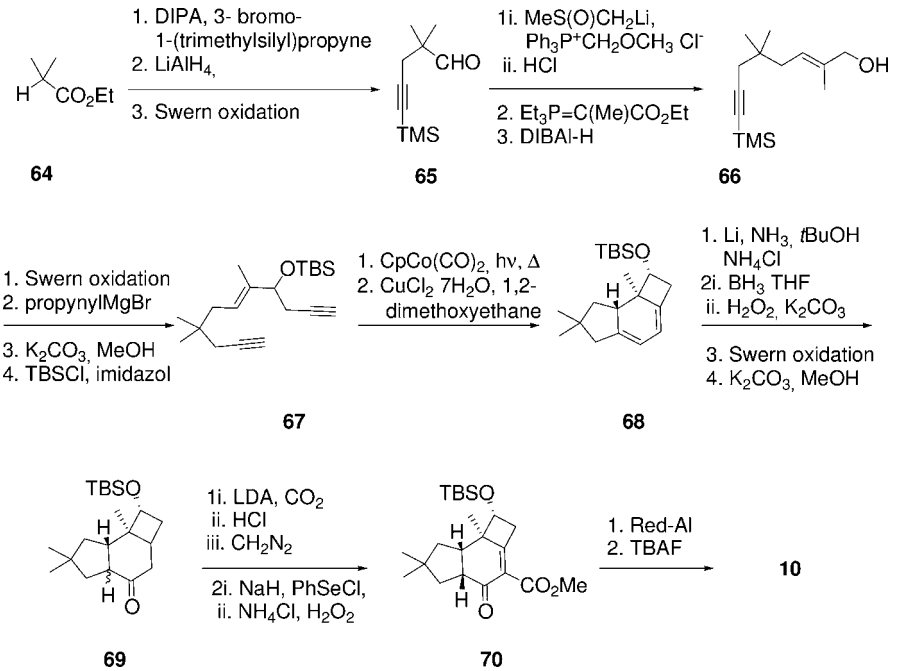
The second racemic formal synthesis of illudol was accomplished by Semmelhack [22]. Again, the ring system was formed in a stepwise manner. Ketone **58** was treated with methyl magnesium bromide and subsequently dehydrated. After ozonolyzis of olefin **59**, the resulting keto aldehyde was subjected to an aldol reaction and treated with *p*TosOH for dehydration. After formation of silyl enolether **60**, a thermally



Scheme 23.3 Semmelhack's formal total synthesis of illudol (**10**).

induced [2 + 2] cycloaddition led to cyclobutene **61**, which was subjected to a highly stereoselective Diels–Alder reaction with **60**. After deprotection, the resulting ketone was reduced and the alcohol benzyl protected to afford compound **62**. Its ester moiety was reduced, the hydroxyl function could be removed via dissolving metal reduction of the corresponding phosphoamidate. These conditions also led to deprotection of the benzyl ether followed by oxidation of the resulting alcohol to ketone **63**. Introduction of the ester moiety and installation of the α,β-unsaturated double bond delivered **57**, an intermediate in the total synthesis of Matsumoto.

The third racemic synthesis of illudol was published by Johnson and Vollhardt (Scheme 23.4) [7]. It was hoped, that stoichiometric $CpCo(CO)_2$ would convert **67** to CpCo-complexed diene **68** by intramolecular [2 + 2 + 2] cycloaddition. All previous examples of these kind of transformations led to more unstrained systems and had less substituents. Moreover, related terminal enediyne cyclizations showed complications according to hydrogen shifts. Alkylation of the enolate of 2-methylpropanoate with 3-bromo-1-(trimethylsilyl) propyne followed by reduction of ester function to the alcohol and subsequent oxidation gave aldehyde **65**. C_1-homologation through sequential Wittig reaction and hydrolysis of the enol ether furnished an aldehyde which was again subjected to a Wittig reaction with high excess of the product



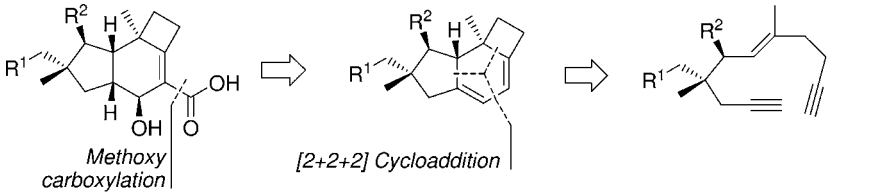
Scheme 23.4 Vollhardt's total synthesis of illudol (**10**).

possessing the desired *E* double bond and subsequent reduction of the α,β-unsaturated ester resulted in alcohol **66**. The obtained compound was then elaborated by standard reactions to enediyne **67** which was then rearranged by $CpCo(CO)_2$ directly to the highly air sensitive diene **68** as the only diastereomer. Remarkably are the efficiency (92%) and stereoselectivity of the process. After Birch reduction, hydroboration/oxidation and Swern oxidation of the newly formed alcohol produced an epimeric mixture of ketones **69** with a *syn* to *anti* ratio of 3 : 2, which could be separated via column chromatography. The subsequent base-catalyzed isomerization of the undesired isomer delivered a *syn* to *anti* ratio of 5 : 1 and after separation, the *syn* isomer was subjected to carboxylation and subsequent esterification with diazomethane. The α,β-unsaturated ester was generated by selenation followed by its oxidation and subsequent elimination to furnish compound **70**. Reduction of the ketone with red-Al gave rise to a mixture of C-4 isomeric diols containing predominatly the desired 4α-OH configuration (37 : 17). Finally, desilylation gave illudol **10**, spectroscopically identical with the naturally occurring material.

Total Synthesis and Configurational Assignment of the Pasteurestins

Retrosynthetic analysis

We started out for the first enantioselective synthesis of the protoilludane skeleton. Retrosynthetically, a *Vollhardt* [2 + 2 + 2] cycloaddition [23] of **71** and **72** should lead

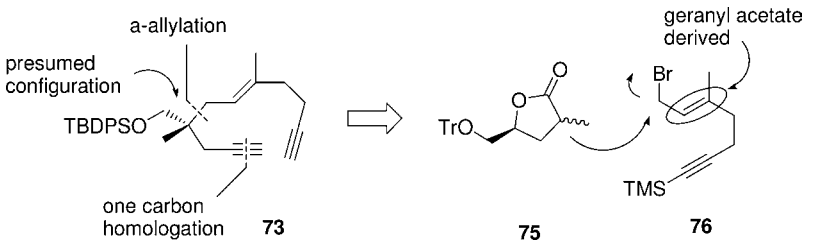


9 R^1 = OH, R^2 = H
8 R^1 = H, R^2 = OH

71 R^1 = OTBDPS, R^2 = H
72 R^1 = H, R^2 = OTBS

73 R^1 = OTBDPS, R^2 = H
74 R^1 = H, R^2 = OTBS

Scheme 23.5 Retrosynthetic analysis for pasteurestin A (**9**) and B (**8**).

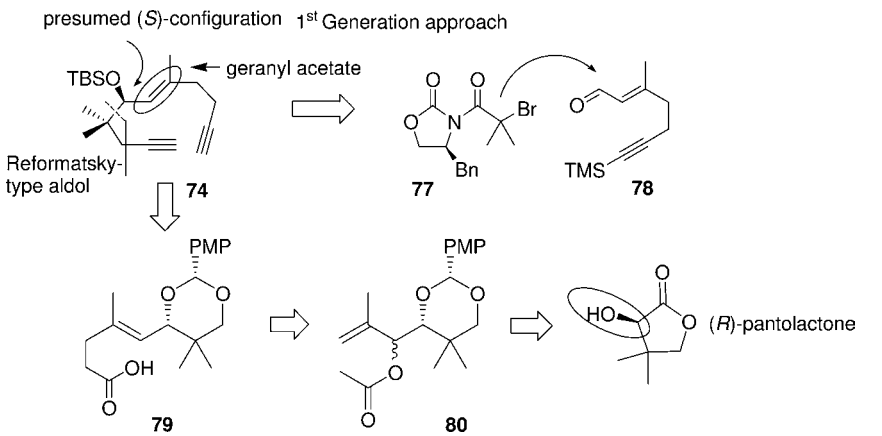


Scheme 23.6 Retrosynthetic analysis of enediyne **73**.

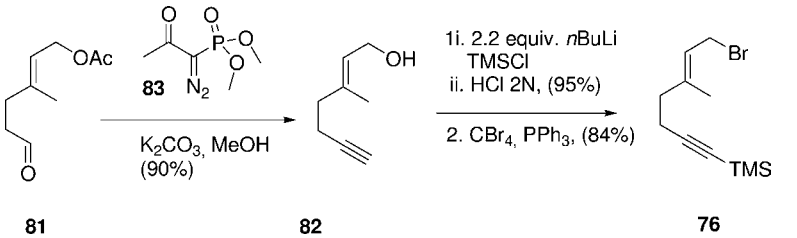
to the tricyclic intermediates **73** and **74** which virtually contain the full carbon skeleton of **8** and **9** (Scheme 23.5).

It was an open question how the existing stereogenic centers in both enediynes would influence the stereochemical course of the cycloaddition.

According to studies by Vollhardt *et al.* regarding the influence of triple bond-substitution in the cyclotrimerization [24], we decided to functionalize the six-membered ring at a later stage in the synthesis. For the construction of enediyne **73** (Scheme 23.6), we employed a substrate-controlled α-alkylation of enantiomerically



Scheme 23.7 Retrosynthetic analysis for chiral enediyne **74**.



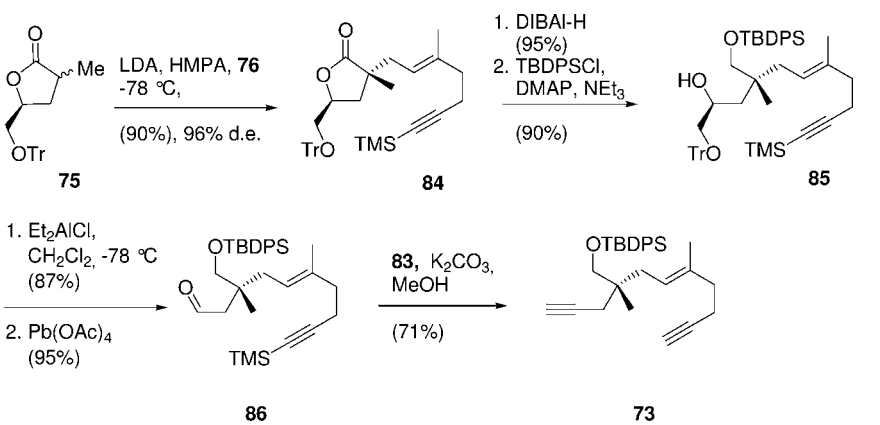
Scheme 23.8 Synthesis of allylic bromide **76**.

enriched butyrolactone **75** with bromide **76**. For optically active pasteurestin B (**8**), we employed a tin-mediated diastereoselective Reformatsky type Aldol reaction (Scheme 23.7) [25–27]. In order to prove the stereochemical outcome of the Reformatsky reaction, enediyne **74** was generated via an unambiguous alternative synthesis starting from (*R*)-pantolactone.

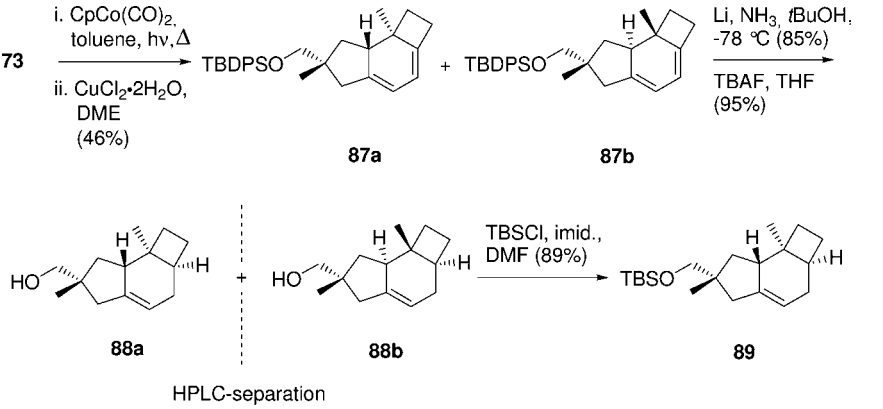
Synthesis of pasteurestin A

Allylic bromide **76** can be obtained in five steps, utilizing the "Bestmann–Ohira reagent", dimethyl-1-diazo-2-oxopropylphosphonate (**83**), as the reagent for the transformation of the aldehyde **81** into alkyne **82** (Scheme 23.8). This was accomplished under *in situ* cleavage of the acetate followed by TMS protection of the triple bond and bromination of the free allylic alcohol.

The synthesis of enediyne **73** was then continued by 1,3-induction upon α-alkylation of butyrolactone **75** which was prepared in two steps from (*R*)-glycidol (Scheme 23.9) [28] with allylic bromide **76** in 71% yield and in high stereo-control (96% d.e.) [29]. Reductive opening of the lactone with DIBAl-H was followed by selective TBDPS protection of the primary alcohol [30] and selective removal of trityl by the use of Et_2AlCl. The resulting diol was cleaved with $Pb(OAc)_4$ (Scheme 23.9). The bulky *t*-butyl diphenylsilyl group was chosen as a stereocontrolling element in



Scheme 23.9 Synthesis of enediyne **73**.



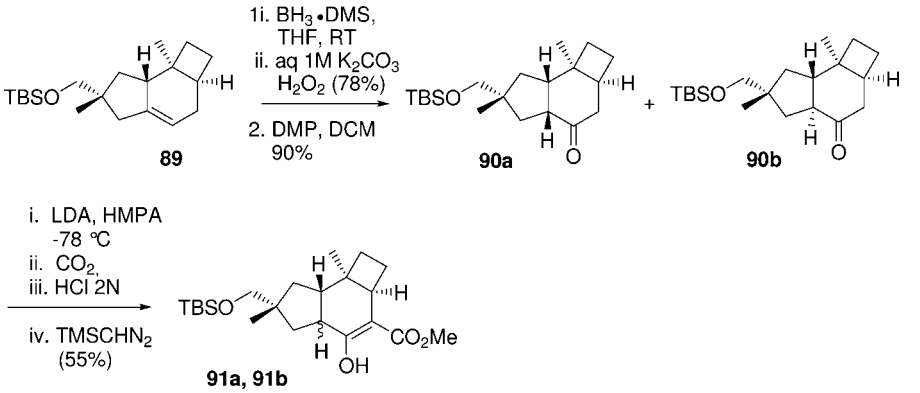
Scheme 23.10 [2 + 2 + 2] cycloaddition and further functionalization.

the [2 + 2 + 2] cycloaddition. One carbon homologation of **86** was then accomplished by the employment of the Bestmann– Ohira reagent **83**.

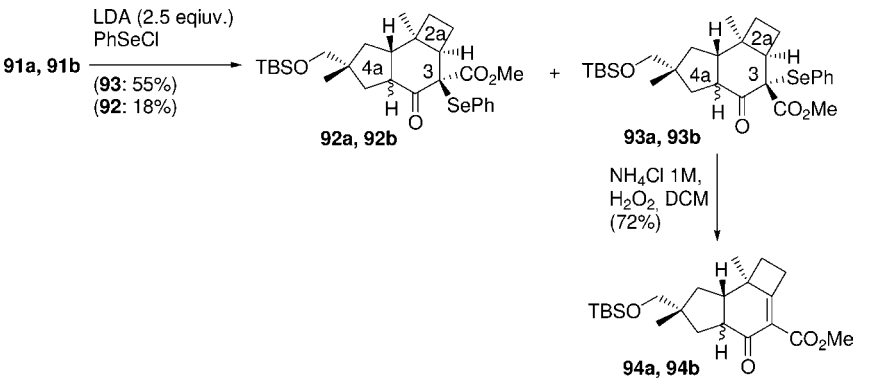
Cobalt-mediated [2 + 2 + 2] cycloaddition in toluene (after reductive demetallation using the relatively mild $CuCl_2 \cdot 2H_2O$) furnished a mixture of diastereomers **87a** and **87b** in a 4 : 3 ratio as the major products. The two extremely unpolar compounds were not separable via column chromatography, and once being decomplexed, proved to be highly air-sensitive. The next problem we had to face was the selective hydrogenation of the C2a–C3 double bond of the dienes (Scheme 23.10). It is very likely that the main reason for a difference in reactivity of the two double bonds in our system is the release of the ring strain of the cyclobutane. When the dienes were exposed to lithium in liquid ammonia, besides the clean regioselective reduction of the C2a–C3 double bond, the phenyl groups of the TBDPS-ether were also reduced. Deprotection using TBAF delivered the primary alcohols **88a** and **88b** which could subsequently be separated via HPLC. The separated products were reprotected as TBS ethers before compound **89** alone was subjected to further functionalization.

Hydroboration represents one of the best methods of hydrating double bonds in an *anti*-Markownikoff manner. All attempts to use sterically hindered agents for the stereoselective hydroboration at C-4 were unsuccessful and led to total recovery of the starting material.

When we employed diborane, we obtained (after oxidation of the resulting alcohols utilizing Dess–Martin periodinane) an inseparable mixture of **90a** and **90b** in a 2 : 1 ratio in 70% yield (Scheme 23.11). Functionalization of C-3 was accomplished by carboxylation of the kinetic enolate anion with carbon dioxide, followed by methylation with TMS diazomethane. Using an excess of base, the desired β-keto esters **91a** and **91b** were obtained in 55% yield in a 4 : 1 ratio. Selenation at C-3 delivered a separable mixture of diastereomers favoring **93** (Scheme 23.12). A mixture of **93a** and **93b** was subjected to H_2O_2 under acidic (aq.·NH_4Cl) conditions and the desired products **94a** and **94b** were obtained in 72% yield. At this stage of the synthesis, the epimers could be separated and only the *syn* epimer was further transformed.

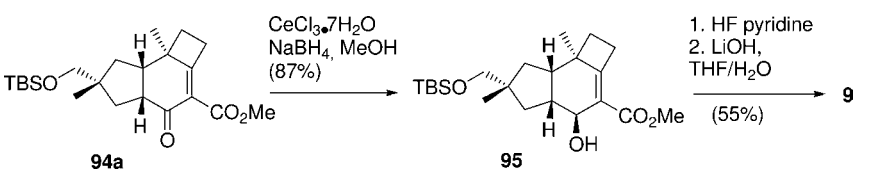


Scheme 23.11 Functionalization of the six-membered ring.

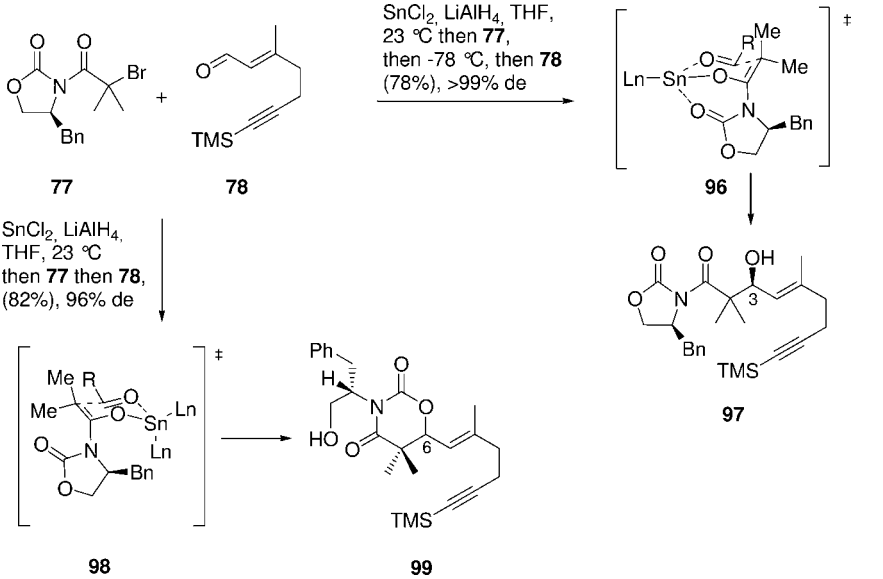


Scheme 23.12 Selenation of **91a** and **91b**.

Stereoselective reduction of the ketone functionality was accomplished by the use of $CeCl_3 \cdot 7H_2O$ and $NaBH_4$ in MeOH delivering a single isomer **95** which was subsequently subjected to HF·pyridine followed by hydrolysis with LiOH in THF/H_2O which gave pasteurestin A (**9**; Scheme 23.13). The determination of the relative configuration was accomplished by ^1H, ^{13}C, and 2D NMR (COSY, NOESY, NMR, HMBC, HSQC), and showed that the data were in accord with those reported. The absolute configuration was proved to be consistent with the patented structure by comparison of the optical rotations.



Scheme 23.13 Endgame to pasteurestin A (**9**).



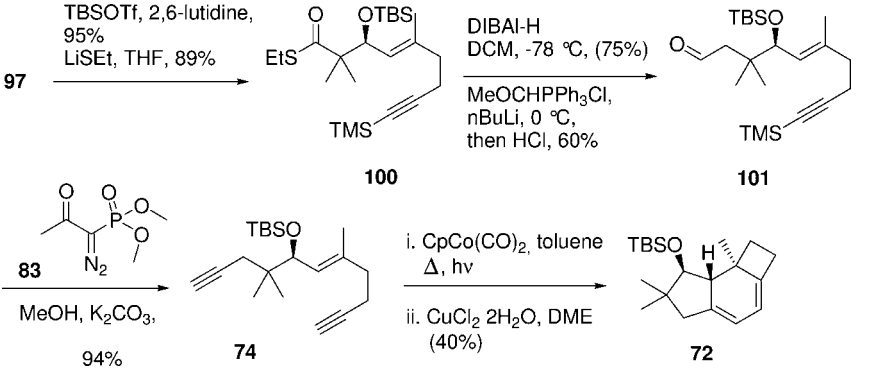
Scheme 23.14 Sn-mediated Reformatsky aldol reaction.

Synthesis of pasteurestin B

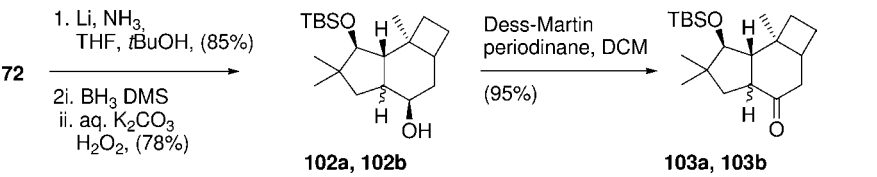
Aldehyde **78** was obtained from the known corresponding alcohol using the Swern protocol. Success in the Reformatsky reaction was ultimately achieved by preparing active tin metal and $AlCl_3$*in situ* from $SnCl_2$ and $LiAlH_4$ in THF according to Harada's and Mukaiyama's procedure [25]. Reduction of the α-bromoisobutyryloxazolidinone **77** by this active Sn^0 solution in THF allowed the generation of a Sn^{II} enolate. At −78 °C, the desired (3*S*)-adduct **97** was obtained in >99% yield under complete retention of the *E*-alkene geometry in 78% yield (Scheme 23.14).

When the whole reaction sequence was performed at room temperature, we obtained the unwanted 1,3-oxazine-2,6-dione **99**. This remarkable temperature effect might be again interpreted in terms of a fully complexed transition state **96** (Nerz–Stormes–Thornton model) at low temperature [31–33] and an uncomplexed Pridgen-type transition state **98** at ambient temperature [34] After purification, the alcohol was protected as TBS-ether and removal of the chiral auxiliary could be accomplished by a method using alkyl mercaptans [35].

Thioester **100** could then be reduced with DIBAl-H to the corresponding aldehyde in 75% yield which was subsequently C_1-homologated by Wittig reaction with methoxymethylidene triphenylphosphine followed by hydrolysis of the enol ether to give aldehyde **101.** (Scheme 23.15). Final aldehyde–alkine conversion using the Bestmann Ohira reagent **83** furnished the precursor **74** ready for [2 + 2 + 2] cyclotrimerization. The absolute configuration of the stereogenic center at C-3 was confirmed by an unambiguous total synthesis of the enediyne **74** starting from, commercially available (*R*)-pantolactone (Scheme 23.7). The obtained enediyne **74** furnished, according to the previously described cobalt-mediated [2 + 2 + 2]



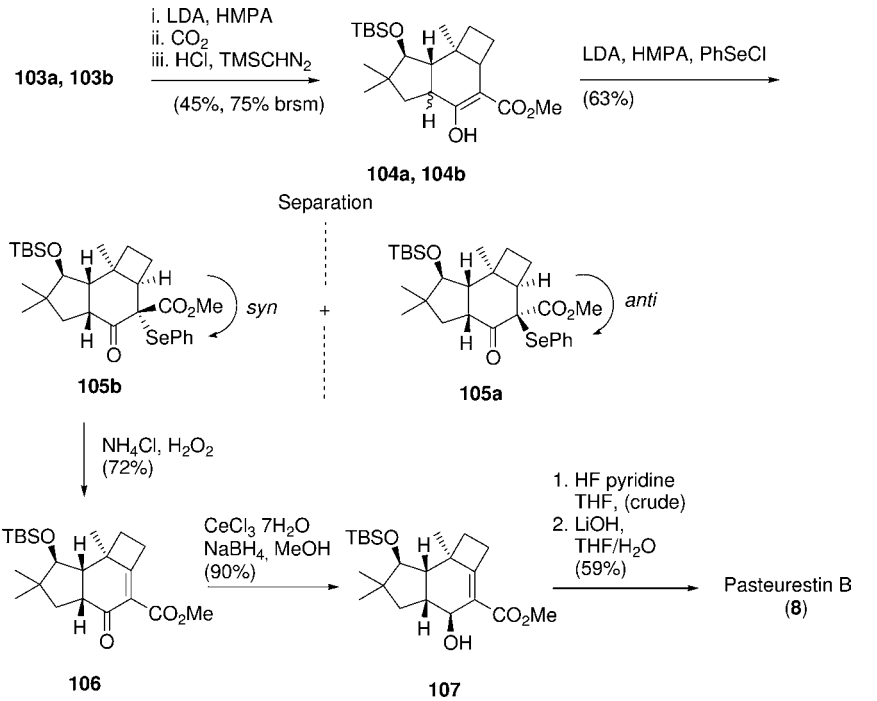
Scheme 23.15 Synthesis of enediyne **74** and cyclotrimerization.



Scheme 23.16 Functionalization of diene **72**.

cycloaddition in toluene, as the major product and after reductive dematallation in a highly diastereoselective manner, diene **72** (Scheme 23.16).

When diene **72** was exposed to Birch reduction, we again observed a *cis* fusion of the 6/4 ring annelation probably due to the low barrier to inversions of carbanions and its assumed greater thermodynamic stability [36]. The C2a–C3 double bond was regioselectively reduced giving the desired monoolefin in 85% yield. Again, the β-face of the alkene is less hindered, and by employment of the parent diborane we obtained, after oxidation, an inseparable mixture of diastereomeric alcohols **102a** and **102b** in a 2 : 1 ratio and 78% yield. Both alcohols were subsequently oxidized with Dess–Martin periodinane to deliver products **103a** and **103b** in 95% yield. Again, functionalization of C-2 in **103a** and **103b** was accomplished by carboxylation of the enolate anion with carbon dioxide, followed by methylation with TMS diazomethane. Double deprotonation of **104a** and **104b** using LDA/HMPA and subsequent selenation also equilibrated the bridgehead proton at C-4a, leading to the *syn* epimers exlusively. The diastereomers could be separated and **105a/b** was subjected to H_2O_2 under acidic (aq·NH_4Cl) conditions to deliver the desired product **106**. The endgame used $CeCl_3 \cdot 7H_2O$ and $NaBH_4$ in MeOH for the reduction which delivered one single isomer **107**. This alcohol was subsequently subjected to HF·pyridine followed by hydrolysis leading to the final product pasteurestin B (**8**) in high yields. The determination of the relative configuration was accomplished by ^1H, ^{13}C NMR, and 2D NMR (COSY, NOESY, HMBC, HSQC) and again matched the data reported for



Scheme 23.17 Functionalization and endgame.

pasteurestin B in the patent. The absolute configuration was proved to be consistent with the patented structure by comparison of the optical rotations (Scheme 23.17).

Conclusion

We have completed the first total synthesis of pasteurestin A (**9**) in 22 steps over the longest linear sequence in 0.4–0.5% overall yield and pasteurestin B (**8**) in 20 steps over the longest linear sequence in an overall yield of 0.8%. By doing so, we could assign the absolute and relative configurations of both target molecules.

Remarkably, screening against a wide variety of bacteria showed that pasteurestin A (**9**) and pasteurestin B (**8**) exhibited micromolar activity and high selectivity for some versatile pathogenic *Pasteurella multocida* strains. The enantiomer of **8** *ent*-(**8**) was also synthesized and did not show any inhibition. Due to functionalization of the six-membered ring at a very late stage, this versatile and flexible approach towards both target compounds allows the preparation of a large number of suitable analogs for further biological testing, which can contribute to the assignment of the mode of action and will help to determine the pharmacophore.

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Index

a

ABC transport proteins 24, 25
– P-glycoproteins 24
abdominal pains 199
absorption, distribution, metabolism, excretion/toxicity (ADMET) 138
academic/industrial environment 136
acceptor/donor amino acid residues 80
acceptor proteins 291
acetyl-CoA 83, 284, 290
O-acetylserine sulfhydrylase 85
Actinobacillus pleuropneumoniae 441
active ligands 333
active pharmaceutical ingredients (APIs) 140, 142, 143
– compounds 141
– descriptor values 143–145
– distribution 142
– origin classes 143–145
active salvage pathway 217
activity-enriched datasets 324, 326
acyliminium ion cyclization method 305
adaptation/stochastic variation 431
adaptive immune response system 163, 164
– regulation 164
adaptive/innate responses 164
adverse drug reactions (ADR) 102
aerobic environment 312
aerobic respiratory chain 359
affinity chromatography 238, 268
African/American trypanosomes 189, 211, 314
African sleeping sickness 87, 212
– causative agents 212
agar plates 348
AGC kinase family 265
– PfPKA-c 265
– PfPKA-R 265
– PfPKB 266
– PfPKG 266
agricultural pests 19
AIDS 278
aldol reaction, Sn-mediated 467
aliphatic compounds 308
alkylaminoalkanethiosulfuric acids 301
allylic bromide synthesis 464
allyl magnesium bromide 460
Alzheimer's diabetes mellitus 241
Alzheimer's disease 264
amebic colitis 184
amide derivatives 304
aminoacetonitrile derivatives (AAD) toxicity 20
amino acids 51, 231, 304, 389
– mutations 253
– residues 228, 231
– rich extension 264
– side-chains 83
α-aminoacrylate intermediate 84
aminoacyl tRNA molecules 400
– inhibitors 382
– synthetases 403
aminoalkanethiosulfuric acids 306, 307, 309
aminoethanethiosulfuric acids 305
anaerobic bacteria 82
anaerobic energy metabolism 91, 92
anaerobic glycolytic fermentations 87
anaerobic mitochondrial electron transport pathway 361
anaerobic nematode 362
anaerobic pathogens 80
anaerobic protozoa 76, 80, 81, 82
– *Entamoeba histolytica* 76, 82
– *Giardia lamblia* 76
– glycolysis 77
– metabolic aspect 76

– *Trichomonas vaginalis* 76, 82
– *Tritrichomonas foetus* 76
analytics/physicochemical assays 112
anaphase-promoting complex (APC) 240
Angiostrongylus costaricensis 343
animal health programs 9
– health market 99
animal parasites 45, 106
– fleas 106
– helminths 106
– *heterakis gallinarum* 45
– *heterodera glycines* 45
– ticks 106
anopheles gambiae 278
anopheline mosquito 177
antagonistic drug action 68
antagonist targets techniques 65
anthelmintic drugs 18, 357, 358, 363, 364
– benzimidazoles 17
– chemical optimization 357
– classes 17
– discovery 9, 18
– macrocyclic lactones 17
– mode of action 18
– nicotinergic agonists 17
– nonspecific resistance mechanisms 24
– resistance issue 17
– specific resistance mechanisms 21
– targets 357
– templates 8
anthelmintics target, GABA receptors 12
antibacterial agent 386, 418
– bacterial expression profiling 381
– cell assay 417
– determination/validation 381
– drug discovery 383, 415, 417, 418
– molecules 384
– MW, distribution plot 148
– therapies 386
Antibacterials Genome Research & Development 377
– impact 377
antibiotics 393, 395, 455
– classes 394
– molecular structures 395
– pathogens 387
– resistant bacteria 375, 386, 393
– status 393
antigen derived epitopes 163
antigen-presenting cell 163, 164, 429
antigen receptor 164
anti-infective agents 75
anti-infective drug discovery, current paradigm 417
antileishmanial drug target 233
antimalarial drugs 178, 249, 250, 253, 254, 310
– activity 453
– drug discovery 380–381
antimicrobial peptides 199
– cathelicidins 199
– θ-defensins 199
– magainins 199
antimicrobial screening programs 376
antimony-containing compounds (pentostam) 212
antinematode drugs 357
– classes 358
antinfectious/parasitic drugs 118
antiparasitic/anti-infective molecular targets 108
antiparasitic compounds 13
– activity 309
– agents 75
– assays 62
– classes 155
antiparasitic drug 3, 4, 5, 10, 45, 60, 61, 62, 75, 94, 117, 119, 170
– approaches 117
– development 239
– discovery 59, 60, 110, 138
– features 62
– identification/validation 59
– research 59
– screening 3
– special challenges 62
– structure 118
– target-based approach 60
– use 4, 117
antiparasitic leads 118
antiparasitic market products 61
antiparasitic screening target 62, 63, 64
– assays 100
– properties 62
antischistosomal compounds 316
antischistosomal drug discovery 302, 310
antisense RNA inhibition 198
antituberculosis (antiTB) drugs 415, 423, 432, 433
– chemical structures 423
– treatment 431
apical secretory organelles 184
apicomplexan parasites 88, 184, 190, 285
– *Plasmodium falciparum* 6, 88
– *Toxoplasma gondii* 88
apicomplexan protozoa 89
apicoplast, 286, 277, 288
– membrane 286, 290

– metabolites 288
– mis-segregation 286
apicoplast-generated pyruvate, *see* FAS II pathway
apicoplast-localized proteins 286
apicoplast-resident pyruvate dehydrogenase 289
apicoplast-resident synthesis 291
Armillaria mellea 458
aromatic compounds 89
aromatic hydroxamates 88
aromatic motif 363
aromatic ring 308
arsenic-based drug 34
artemisinin derivatives, antischistosomal activity 309
asexual blood-stage malaria parasite 178, 180
aspartic protease inhibitor, activity 198
ATP-binding proteins 229
– active site 263
– binding site 229, 241
– dependent ligases 218
– dependent phosphofructokinase 77
– dependent reaction 86
– grasp proteins 218
– homologs 218
attenuated rabies vaccine 169
– vaccine strain 168
attenuated strains, infection 167
aurora kinases 239
– inhibitors 239, 240
auramine-rhodamine fluorescent staining 425
auxillary pathways 286

b

β-lactam antimicrobials 399
B-specific inhibitors 186
β-tubulin 12
– sequence 26
Babesia-derived proteases 183
Babesia divergens 169
– infections 167
bacterial antigens 389
bacterial enzyme 77
bacterial genome 376
– sequence 442
bacterial pathogens 386
– infections 384, 387, 393
bacterial ribosome 399
bacterial species 163
bacterial toxins 391
bacterium-type FAS II 290
basidiomycete sesquiterpenoids 456
BCUT matrix analysis 108
benzimidazole 23
– binding site 23
– resistant nematodes 23
binding site residues 263
bioactive substances 62, 125
biochemical/cellular assays 99
biocide compounds 128
– ionophores 128
– uncouplers 128
bioinformatic analysis 50
– database searches 50
– multiple sequence alignments 50
– tools 65, 90
bioinformatic sequence 48, 51
– analysis 51
biological assay systems 99, 106
biological sequence data, *see* Genomic filtering
biological systems 219
biology-based intellectual property 8
biology-driven drug discovery 124
bioscreen scores 367
biosynthetic process 82
biotinylated fluorophosphonate probe 184
bipartite targeting domain (BTD) 286
Birch reduction 462
black widow spider venom 345
BLAST alignments 50
blood-stage parasites 179
blood-stage schizont rupture 180
bloodstream/cerebrospinal fluid 228
bloodstream form (BSF) trypanosomes 240
bovine cells 190
bovine pathogen 441
bovine respiratory disease (BRD) 453, 455
broad-spectrum antibiotics 401
– carbapenem 401
– ceftaroline 401
– cephalosporin 401
– tigecycline 400
broad-spectrum antiparasitic drugs 119
broad-spectrum protease inhibitors 179, 183
bronchocentric granulomatosis 425
bronchovascular/mediastinal structures 429
2-(butylamino)-1-phenyl-1-ethanethiosulfuric acid 308

c

Ca-dependent kinase activity 267
Caenorhabditis elegans 20, 22, 46, 94, 123, 346, 347, 348, 350, 352
– emodepside action 346
– latrophilin receptor 347

– L4 larval stages 348
– mutagenesis protocols 22
– mutant strains 22
calcium-activated K^+ signaling pathway 352
– voltage-gated K^+ channel 19, 350
calcium/calmodulin antagonists 267
calcium-dependent channels 397
calcium-dependent protein kinase family 267
– PfCDPK1 267
calpain-like cysteine proteinases 185
cAMP-dependent pathway 265
cancer chemotherapy 249
cancer therapy, inhibitor 212
CAP3 software 48
cardiovascular disease 249
casein kinase 1 (CK1) enzymes 237
casein kinase family 268
– PfCK1 268
catalyst-mediated processes 305
catalytic hydrogenation 459
cathepsin L-like protease 189
cattle pathogen 183
– *Babesia bovis* 183
CDK-activating kinase (CAK) 235, 262
– post-translational control 235
– schematic presentation 235
CDK inhibitors 236, 237
– flavopiridol 236
– olomoucine 236
– phosphorylation site 263
cDNA 182, 344, 385
cell-binding domain 180
cell-mediated immunity 278
cell permeable peptidyl ketone inhibitor 186
cell surface receptors 163
cellular assays 435
cellular immune response 162
cell wall synthesis 402
central nervous system toxicity 25
cGMP signaling pathway, binding sites 266
Chagas' disease 33, 34, 35, 36, 180, 185
– acute 35
– chronic 35
– indeterminate 35
Chagas' drugs 214
– benznidazole 214
– nifurtimox 214
chemical parameters 129
– aqueous solubility 129
– volatility 129
chemoinformatic/molecular modeling techniques 47
chemoinformatic tools 128, 359
chemotherapeutically controlled infection 166
chemotherapeutic drug 170
chiral enediyne, retrosynthetic analysis 463
Chlamydomonas reinhardtii 238
chloride-gated ion channel 91
chlorinated hydrocarbons/organophosphates 4
chloroplast-like malarial organelle 178
chorismate synthase 89
CK enzyme 238, 268
– inhibitors 238
coagulase-negative staphylococci 387, 393
coagulation systems 163
coccidiostatic drugs 166
cognate interactions 162
compound logistics 111, 112
– handling/supply 111
computational algorithm analysis 390
computational/experimental approaches 376
computer-aided modeling 401
computer-based methods 323
contaminated food 186
Cooperia oncophora 344
cost-benefit analysis 6
cowpox induced immunity 167
cross-species vaccination 167
C-terminal domains 181
– inhibitory regions 268
– pyruvate/PEP binding site 79
cyclic octadepsipeptides 351
cyclin-dependent kinases 234
cyclooctadepsipeptides 339, 344
– mechanisms of action 344
– PF1022A 20
cyclohexane ring 303
cysteine/aspartic proteases 178
cysteine biosynthetic pathway 76, 82, 83, 84
cysteine endopeptidases 178
cysteine protease family, schematic presentation 196
cysteine proteases (E64) 182, 185, 186, 188, 198, 314
– inhibitors 181
cysteine proteinase genes 185, 186
– EhCP1 185
– EhCP2 185
– EhCP5 185
cysteine synthase 82
cystic fibrosis 404
cytokines gamma-interferon γ(-IFN) 164
cytoplasmic acidic vesicles 345
cytosolic serine protease 198

d

daptomycin 397
datasets prefiltering 329
DBL-EBP family 179
DDT 6, 165
de novo synthesis 290
dense granule-like organelles 179
1-deoxy-D-xylulose-5-phosphate reductoisomerase 90
Dess–Martin periodinane 465
detoxifying enzymes, P450 21
2D gelatin gel electrophoresis 182, 446
diabetes 177, 229, 239, 241, 264
diagnostic methods 381
diastereomeric salt/reacylation 305
diazoacetyl-norleucinemethylester 198
dideoxy chain termination sequencing technology 443
Diels–Alder reaction 461
dimethylallyl pyrophosphate 289
dimethyl sulfoxide 110
– stock solutions 110, 111
discovery paradigms 7
discovery synergies 8–10
disease-focused treatment 455
disease-relevant parasitic stages 124
disulfide bridge 52
dithiol-containing medium-chain fatty acid 290
diverse assay technologies 112
– cellular assays 112
– enzymatic 112
– ion channel 112
– microbial parasite assays 112
– protein-protein binding 112
DNA microarrays 381, 385
– oligonucleotide-based arrays 385
– PCR product-based arrays 385
– technology 385
DNA replication 263, 403
– bacterial DNA gyrase 403
– topoisomerase IV 403
DNA sequences 47, 169, 380, 385, 446, 447
– amplification/sequencing 445
– metabolism 389
– vaccination 388
DNDi 36, 38, 41
– data management 41
– decision matrices 40
– discovery manuals 38
– lead optimization 36
– schematic presentation 40
docking experiments 48, 53, 329
dopaminergic receptors 327
double-stranded RNA (dsRNA) 66
– interference 211
DOXP synthase (Dxs) 289
D-phenyllactic acids 339
Drosophila melanogaster 46, 50, 51, 53, 54, 61, 62, 64, 66, 123
– genome 64
– phenotypic effect 61
drug addiction gene 168
drug design 8, 70
– mechanism-based 119
– process 324
– structure-based 8
drug discovery process 4, 8, 10, 17, 25, 45–48, 59, 119, 135, 136, 182, 302, 334, 421, 434
– contribution 25
– mechanism-based approaches 8
– mode of action 18
– nonspecific resistance mechanisms 24
– specific resistance mechanisms 21
– key technologies 45
– schematic presentation 48
drug-mediated interference 183
drug-resistant parasites 165
– nematodes isolation 23
drug-resistant strains 21
– drug-resistant TB 417
– MTB strains 416
drug metabolism 24
– cytochrome P450 24
drug resistance 249
– genes 22
drug targets 3, 63, 213, 301
– validation 213
DV-located metalloprotease 178
Dxr inhibition 289
dynamic polymers, *see* microtubule

e

ectoparasitic field 62, 129
ectoparasiticides/agrochemical insecticides 6, 129, 146, 147
– ivermectin 6
egg-laying muscles 348
Eimeria parasites 166
electron transport chain 360, 362
electrophysiological/binding studies 344
emodepside 343
– anthelmintic activity 343
– interactions 346
– parasites 23
– receptor 349
– SLO-1 349
– strains 349

EMS chemical mutagenesis 349
endocytotic vesicles 238
endo/ectoparasites 50, 51, 104
– orthologous sequences 50
endogenous glutamate transmitter 94
endothelial cells 163
energy metabolism 77, 359
energy-transducing mechanism 360
engineering industries, car industry 136
enoyl-acyl carrier protein (ACP) reductase 290
Entamoeba histolytica 6
– enzyme 77
– genome 85
– pathogenicity 185
enumeration processes, impact 328
enzymatic assays 181
enzymatic synthesis 288
epoxide cysteine proteinase inhibitor 185
erythrocyte-binding ligands 179
erythrocytic cycle 177
erythrocytic schizogony 262
Escherichia coli 289
EST sequences 45, 50
eukaryotic cells 18
eukaryotic protein kinases (ePK) 228
experimental methods 138
expressed sequence tags (ESTs) 48
extrapulmonary tuberculosis 429

f

FabF/FabH inhibitors 402
FACS analysis 388
farnesyl pyrophosphate synthase (FPPS) 288
– inhibitor 289
FAS II pathway 289
– enzymes 290
– gene 290
– inhibitors 290
fatty acid/lipoic acid biosynthesis 289
– pathway 402
Fd redox system 291
Fe-binding protein 189
fermentation products 3
ferredoxin-linked enzyme 76
ferredoxin redox system 291
flavin-containing glycerol-3-phosphate dehydrogenase 87
flavin-containing trypanothione reductase 86
flock immunity, development 167
fluorogenic peptides 181
FMRFamide-like peptides (FLPs) 13
focused libraries 110
fosmidomycin/NADPH, H-bond network 92
free-living amoeba, *Naegleria fowleri* 77
Freunds complete adjuvant (FCA) 161
fructose 6-phosphate 77
fruitfly sequence 53
function-based screens 124

g

GABA-gated chloride channel, fipronil 64
GABA receptor 21, 344
GAL4 activator 66
GAL4 driver 66, 68
GAL4 enhancer trap insertion database (GETDB) 68
GAL80 repressor 66
γ-proteobacteria 441
gap-closure 441, 445
– PCR-based strategies 445
– workflow 441
gastrointestinal mega-syndromes 180
gastrointestinal nematodes 343
gastrointestinal/respiratory systems 187
gastrointestinal tract physiology 126
gene expression systems 379
GENESWITCH system 66
genetic algorithms 332
genetic interaction 65
genome analysis 375, 389
genome-based technologies 376, 384
genome evolution 376
– gene gain 376
– gene loss 376
genome sequences 375, 381, 388
– analysis 381
genome sequencing projects 212, 384, 441
genome-wide protocols 12
genomic filtering 47
genomics-based approaches 11
genomics gap closure 443
genomics Genlight software 48
genotoxic drugs 34
genotypic/phenotypic data, statistical analysis 142
genotyping method 385
Giardia lamblia 199
– infection 168
Giemsa-stained smears 183
global expression analysis 382
globular proteins 217
glutamate-gated chloride channels (GluCl) 21, 64, 93
glutathione reductase 312
glutathionylspermidine synthetase (GspS) 213

glycolysis-derived phosphoenolpyruvate (PEP) 288
glycopeptides 397
glycosomal pyruvate phosphate dikinase 79
glycosylphosphatidylinositol anchor 185
GMGC kinase family 256
– Pfcrk-1 263
– PfGSK3 264
– Pfmap-1 256
– Pfmap-2 256
– Pfmrk 262
– PfPK5 263
– PfPK6 262
golgi-associated proteins 238
– cisterns 181
– snapin 238
G protein-coupled receptor (GPCR) 344, 351
GPx-type peroxidases 218, 220
gram-negative pathogens, MRSA 400
– bacteria efflux 404
gram-negative species 379
gram-positive model organism 378
– *Bacillus subtilis* 378
gram-positive multidrug-resistant pathogens 397
gram-positive pathogens 379, 383, 395, 396, 398, 403
– *Staphylococcus aureus* 379
– streptococci 398
growth-inhibiting compounds 419
GSH reductase, structures 86
GSH system 213
– derivatives 219
guinea pig 422
gut-dwelling parasitic nematode 359

h

Haemophilus influenzae 397
Hageman factor 163
HAP pathogen 442
hard-to-treat diseases 211
– African sleeping sickness 211
– Chagas′ disease 211
H-bond acceptors/donors 109, 138, 142
H-bond network 83
HC110-R disposition, schematic view 345
heat-killed bacteria 161
HeLa cells, CP65 182
α-helical carboxy-terminal lobe 229
Heligmosomoides polygyrus 342
helminths 91
– drug initiative 7, 315
– infections 166
– parasitic 314
hematin accumulation 307
heme pathway 284
herpes virus of turkey (HVT) 167
heterocyclic moiety 363
heterocyclic ring system 363
heterodimeric receptor complexes 67, 83
high-activity anti-retroviral therapy (HAART) 188
high-dose infection 167
high-level macroschizont-specific expression 196
high-performance computing (HPC) platforms 324
high-throughput liquid chromatography (HPLC) 111, 141, 465
– mass spectroscopy 111
high-throughput molecular docking methods 46, 136, 285, 291, 323, 325, 326
– approaches 54
– bioinformatics 46
– method 335
– process 330, 333
– relevant binding pockets 327
– schematic representation 326
– workflow 328
high-throughput screening (HTS) 61, 70, 106, 179 313, 323, 302, 383, 386
– assay 63, 383
– compatible enzymes 420
– libraries 110, 324
– methods 455
– screening 63, 64
– systems 13
– technology 323
high-throughput technologies, pyrosequencing 47
hit-to-lead process 109
HIV-infected patients 170
holoenzyme complex 87
homodimeric enzyme 78, 80
homodimeric PPDKs 79
homologous enzymes 185
Homo sapiens 278
horse gastrointestinal nematodes 18
host cell hemoglobin 178
host defense mechanisms 159
HRD motif 231
human African trypanosomiasis (HAT) 33, 34, 35, 39, 240, 189, 301
human cysteine protease cathepsin 182
human embryonal kidney cell assay (HEK293) 328, 346
human filariasis 4

human health drug discovery 59, 61, 62, 63
– simulation 62
human kinase 251, 254
– drug discovery efforts 251
– inhibitors 254
human necrotizing granulomata 422
human parasite-targeted screening stream 9
human target proteins 327
human trematocidal compound 120
human vaginal epithelial cells 182
human/veterinary drugs 153
– health 125
– helminth infections 17
hybridization reaction 385
hydrindane moiety 459
hydroperoxide metabolism 213, 215
hydroperoxides detoxification 211
hydrophilic drugs 432
hydroxamate group 91
hydroxamic acid derivatives 402

i

illudoid family 457
illudol 460, 461, 462
– Matsumoto′s synthesis 460
– Semmelhack′s synthesis 461
– Vollhardt′s synthesis 462
imaging mass spectrometry (IMS) 434
immune response 164, 221, 424
– T-helper 1 cells (Th-1) 164
– T-helper 2 cells (Th-2) 164
immune system 197
immunoelectron microscopy 181
immunogenic epitopes 164
immunology 161
– adaptive immunity
– natural immunity 162, 164
immunomodulating cytokines 159
immunotherapy/vaccine development 387
incubation period 163
industrial drug discovery 136
infectious diseases 117, 165
– chemotherapeutic treatment 165
– control 165
inflammatory disorders 229
inflammatory reaction 427
inhibitor binding sites 84
inhibitory ionotropic receptors, GluCls 93
inhibitory scaffolds 254
innate immune responses 163
in silico gap closure 444, 445
– MUMmer dot plot 444
– pharmacophore approaches 328
in silico screening methods 54, 324, 328, 334
– homology modeling 54
– molecular docking 54
– sequence analysis 54
in silico tools 121
– molecular docking 121
– molecular modelling 121
– pharmacophore design 121
– virtual screening 121
integral membrane proteins 179
– AMA1 179
– PTRAMP 179
intellectual property 111
interleukin 2 163, 164
intra-abdominal infections 400, 401
intracellular apicomplexan parasite 278
intracellular efflux pumps 404
intra-erythrocytic growth 177
introns 25
in vitro antischistosomal activity 307
in vitro assay 107, 323, 336
– derivatives 127
in vitro bactericidal activity 398
in vitro/in vivo assays 435
in vitro kinase activity 269
in vitro lipophilicity activity 365, 367
– correlation 365, 367
in vitro therapeutic index 255
in vivo antiparasite screens 59
in vivo biological activity 125
in vivo pharmacodynamic studies 122
in vivo screens 7
iron-sulfur clusters 361
isoleucyl-tRNA synthetase (IleS) 382
isopentenyl pyrophosphate 89
isoprenoid biosynthesis pathway 90, 288, 291
– steroids/dolichols/quinones 89
isoquinolinesulfonamide compound 268
ivermectin/moxidectin treatments 24
ivermectin-resistant nematodes 339

k

killed vaccines/adjuvants 161
kinase activation cascade 256
kinase inhibitors 251
kinetoplastid parasites 6, 197, 234, 312
– *Cryptosporidium parvum* 6
– *Entamoeba histolytica* 6
– *Giardia lamblia* 6
– *Leishmania donovani* 6, 212, 312
– *Trypanasoma cruzi* 6
– *Toxoplasma gondii* 6

– *Trichomonas vaginalis* 6
– *Trypanosoma brucei* 6, 312
kissing bug 35
knockout experiments 60
knockout mutants 65

l

Lactarius atlanticus 459
large-scale interactomic maps 387
latrophilin 344
– G protein-coupled receptors 348, 357
α-latrotoxin signaling 345
lead compounds 108
lead discovery project 107
lead optimization 126, 130, 362
– factors 130
– process 101
– projects 127
– workflow 126, 127, 128 130
Leishmania infantum infection 169, 170
Leishmania mexicana parasites 168
– deletion mutants *233*
– LmxMPK4 232
Leishmania promastigotes 197
leishmaniasis chemotherapy 197
leukocytes 163
levamisole-resistance genes 22
– acr-8 22
– lev-1 22
– lev-8 22
ligand-based virtual screening approaches 323, 329
ligand binding affinity 332
ligand-gated anion channels 21
ligand-gated ion channel, schematic representation 94
light microscope 161
lipoic acid synthesis 290
– 3D structures 290
– LipA 290
– LipB 290
– synthase 291
lipoamide dehydrogenase 219
lipophilic drugs 364
Litomosoides sigmodontis 343
long-chain fatty acid esters 459
low-affinity inhibitors 262
low-dose infections 166, 167
low-throughput systems 7
α-LTX-HC110-R 346
L-serine *O*-acetyltransferase 83
lungworm parasites 168
lymphoproliferative disorder 190
lysosome-like digestive vacuole 178

m

macrocyclic lactones 9, 12, 21, 25, 94, 147, 340, 358
– antinematodal drugs 94
macroschizont membrane 196
malaria 183, 250, 277, 278, 279
– chloroquine 277
– drug discovery 251
– drugs/drug combinations 279–284
– drug resistance 253
– eradication program 165
– infection 166
malaria kinase drug discovery 254, 255
– considerations 254
– coupling target 255
– homology 251
malaria kinome 249
malarial casein kinase activity 268
malarial infection 180
malarial kinases 249, 251, 252, 253, 254, 255, 256
– characteristics 257–261
– drug discovery 252
– drug targets 257
– inhibitors 251, 255
– promiscuity 255
– schematic presentation 252
– structural considerations 251
malaria transmission 267
malaria vaccine initiative (MVI) 34
mammalian cell-based assays 255
mammalian cell lines (COS-7/HEK-293) 345
mammalian cells 25, 240
mammalian glutathione (GSH) reductase 87
mammalian glycogen synthase kinase (GSKs) 264
mammalian homologs 218
– glutathione reductase 218
– thioredoxin reductases 218
mammalian-infective stage 233
mammalian latrophilin receptor 345
mammalian multidrug resistance proteins 25
– MRP 2 25
– MRP1 25
– MRP3 25
mammalian protein kinases, inhibitor research 241
mammalian redox mediator glutathione 211
mammalian species 187
Mannheimia haemolytica 443
– genome sequencing 443
– M7/2 genome 447
– PHL213 441

– schematic map 448
– strains 444, 456
Mannich bases 219
– inhibitors 312
Marek's disease virus 167
Mastomys coucha 343
maternal immunity 167
matrix metalloproteases (MMPs) 190
mitochondriate protozoa 76, 77, 78
– *Entamoeba histolytica* 78
– Giardia 78
medicinal chemistry 10
Medicines for Malaria Venture (MMV) 34, 179, 289, 301
membrane-bound enzymes 69
membrane-bound receptors 164
membrane-bound subtilisin-like protease, SUB2 179
MenB genome sequence 388
meningococcal antigens 389
MEP pathway, enzymatic steps 91
MEROPS database 190
messenger-independent, serine/threonine protein kinases 237
metabolic pathways 75, 212, 277, 278, 285, 286, 287, 387
– schematic presentation 287
metabolic stability 324
metabolite transporters 286
metacaspase null parasites 198
meta-genomics databanks 395
metalo-dependent cysteine protease 188
metazoan pathogens 19
methicillin-resistant *Staphylococcus aureus* (MRSA) 398, 431
methionyl tRNA synthetase (MetRS) inhibitors 384
methionine-γ-lyase (MGL) isoforms 82
methylene-containing derivatives 363
methyl group donor 51
– S-adenosyl-L-methionine 51
mevalonate pathway 89
microaerophilic/anaerobic conditions 361
microarray analysis 382
microbial genomics 380–381
– sequences 442
microbial surface components 391
microbiological pathogens 160
microbiological techniques 386
microcrystalline cellulose triacetate (MCTA) 305
microdialysis 433
microtubules 18
minimal chronic inflammatory cell 427
minimum effective concentration (MEC) 359
minimum inhibitory concentration (MIC) 455
Ministry of Health in Malaysia 36
mitochondria 360
– electron transport system 88
mitochondrial respiratory systems 360
– respiratory chain 361
mitochondrial tryparedoxin 216
mitogen-activated protein (MAP) kinase 229–234
– homolog 233
– schematic presentation 231
– signal transduction cascade 231
– signaling cascade 256
– structure 232
mitosis regulators, *see* aurora kinase
mitotic cyclin degradation 240
molecular assays/cellular models 104
molecular/biology techniques, 46
– array 431
– genomics 46
– safety assays 103
– scaffold 41
molecular docking experiments, schematic presentation 48
molecular modeling techniques 455
monoclonal antibodies 392
– aurexis/tefibazumab 391
monomeric trypanothione synthetase 86
moonlighting proteins 292
multidrug-resistant (MDR) microorganisms 404
– CL347 099 24
– verapamil 24
multidrug-resistant bacterial pathogens 376
multidrug-resistant pathogens 397, 404
multidrug-resistant strains 309
multi-objective lead optimization process 122
multiple sequence alignment 52
– EST sequences 52
– SAM synthetase sequences 52
multiresistant bacterial pathogens 387
multi-step enzymatic reactions 76
MUMmer dot plot 444
Mycelia sterilia PF1022 340
Mycobacterium tuberculosis (MTB) 91, 160, 381, 415, 416
– infection 170
– light microscopy 416
– scanning EM 416

n

N-acetylglucosamine-enolpyruvyl reductase (MurB) 402
N-acetylneuramic acid 388
NADH-fumarate oxidoreductase 362
NADH-fumarate reductase system 92, 93
– schematic presentation 93
NADPH 291
– reoxidation 361
N-alkyl-2-amino-1-phenyl-1-ethanethiosulfuric acids, antischistosomal activity 308
narrow-spectrum antibiotics 12, 386
natural ectoantiparasitics/anthelmintics 149
natural immunity 162
N-methyl-D-leucine 344
N-methyl-L-leucine 340
N-S-blocked glutathione monoester/diester derivatives 219
N-terminal domain 266
– lobe 251
N-terminal nucleotide binding site 79
neglected tropical diseases (NTDs), schistosomiasis 301
nematode genes 11
– larvae 364
nematode ion channels 357
– 4-aminobutyric acid (GABA) 357
– glutamate-gated chloride channels 357
– nicotinic acetylcholine receptors 357
nematode nervous system 19, 94
nephelometric method 155
neuromuscular junction 19, 349
neuronal/neuromuscular transmission 93
neuron depolarization 94
neurotransmitter receptors 19
– ligand-gated anion channels 20
– ligand-gated cation channels 19
NeuTec pharma 391
nicotinergic agonists 17
– imidazothiazoles 17
– tetrahydropyramidines 17
nicotinic acetylcholine receptors (nAChRs) 12, 19
– agonists 340
nicotinic cholinergic agonists 4
nicotinic receptors 20
NIH Chemical Genomics Center 313
NIMA kinase 264
– family 264
– Pfnek-1 264
– Pfnek-3 265
Nippostrongylus brasiliensis 342
nitrogen-containing bisphosphonates 77
nonATP-binding pockets 252
Non-Governmental Organization (NGO) 302
non-live vaccines 168
non-natural compounds, property values 149
non-necrotizing granulomata 426
non-peptide agonists/antagonists 13
non-photosynthetic plastid-like organelle 89
non-replicating persistence model (NRP) 435
nonmethionine aminopeptidases 178
nontoxic cysteine proteinase inhibitors 186
nontoxic derivative anhydrotetracycline 379
nosocomial pathogens 384
– *Staphylococcus aureus* 378
novel antimalarials approaches 291–292
nuclear magnetic resonance (NMR) 111
nucleated cells 163
nucleic acid metabolism 377
nucleic acid synthesis 211
nucleotide binding oligomerization domain (NOD) receptors 164
null mutants 232

o

Oesophagostomum dentatum 363
optimization process 121
optimization programs 125
orphan protein kinases 268
– PfPK7 268
– PfPK9 269
orphan receptor, HC110-R 344
orthologous genes 65
orthologous proteins 50
osteoporosis 289
oxidative stress 214, 217

p

polar surface area (PSA) value 105, 142
– molecular parameters 105
O-phosphoserinesulfhydrylase 85
palladium complexes 219
– Pd(5-nitrofuryl-3-acroleine thiosemicarbazone)2 219
PAMPA double-sink protocol 141
panton-valentine leukocidin (PVL), 396
pantothenate kinase 418
papain family 186
– proteins 179
para-amino salicylic acid 422, 423
parasite encoded peptidases 190
parasites hydroperoxide metabolism 216
parasite-infected cell 190
– cultures 266
parasites/infective bacteria 106
parasite membrane 196
parasite metabolism 17

parasite proteins 3
– proteases 183
parasite redox pathway 312
– balance 219
parasite-selective CDK inhibitors 227, 237, 238, 241
parasite-specific molecular targets 211
parasite-specific pathway 213
parasite-specific processes 285
parasite-specific protein, tryparedoxin 213
parasite target proteins 50
parasites selective drug targets 75
parasitic cysteine proteases 185
parasitic diseases 160
parasitic helminth 360
parasitic infections 162
– infectious diseases 45
– material 126
– vaccination strategies 162
parasitic model systems 61
parasitic nematodes 12, 17, 19, 23, 350, 357, 362
parasitic protozoa 45, 241, 261
– status/validation 177
parasitic vaccines 168
– species 11
parasitophorous vacuolar membrane (PVM) 269
– vacuole 267
passive immunoprophylaxis 390
Pasteurella aviseptica 160
Pasteurellaceae-specific antibiotics 455
Pasteurella multocida 453
– strains 469
Pasteurella-specific natural products 455
pasteurestins, synthesis assignment 462
pathogen associated molecular patterns (PAMP) 164
pathogen-derived molecules, antigens 163
pathogen-host interactions 375
pathogenic bacteria 378, 376, 381, 388
pathogenic processes 375
pathogenic species 212
pathogenic tubercle 160
pathogenomics, definition 375
pathogen recognition receptor (PRR) 163
pathophysiological process 427
paullone-based TryS inhibitors 220
penicillin-binding proteins (PBPs) 402
penicillin-resistant streptococci 396
peptide deformylase 402
– inhibitors 418
peptide trypanothione 218
peptidoglycan chains 398
peptidoglycan synthesis 419
peptidyl transferase 382
permeability/lipophilicity values 135, 154
peroxide bond 311
peroxiredoxin-type tryparedoxin peroxidases 214
pesticidal entities 363
PF1022A structures 340, 341
– anthelmintic activity 341
– discovery/synthesis 340
Pfmrk, autophosphorylation 262
PfPKA-R protein 265
PfPK5 inhibitors 263
PfPK7 drug design 269
phagocytic cells 197
pharmaceutical research 135
pharmacogenomic antibiotic screens 394
pharmacokinetic data 434
pharmacokinetic profile 131
pharmacophore 325
– approaches 325
– based focused libraries 109
– based virtual screening 328
– 3D geometric arrangements 325
pharyngeal muscle 20
pharyngeal neuromuscular junction 350
phenotype databases 50
phenotypic classes 139, 141
– determination 141
– properties/assignment 139
phenotypic drug resistance 431
phenotypic/genotypic tests 385
phenotypic screens 419, 421
phenyl-thiazolylurea-sulfonamides 403
phenylalanyl-t-RNA synthetase (PheRS) 383
– inhibitor 382, 403
phosphatase catalytic domains 228
phosphinic acid derivatives 218
phosphinic amides 313
phosphohydroxypyruvate intermediate 83
phosphoinositide interaction 266
phosphonohydroxamate compounds 91
phylogenetic analysis 445
phylogeny/experimental data 380
– expression profiles 380
– proteome data 380
physiology-based experiments 126
physiology-based screens 125
plant-derived metabolic pathways 286
plant enzyme 77
plant-like MKPs 228
Plasmodium falciparum 6, 178, 253, 284, 289, 309
– chorismate synthase 90

– extrachromosomal DNA 285
– genome 285
– homology model 90
– infected RBCs 265
plastid-like organelle, *see* Apicoplast
plastid-localized targets 285
plastid-specific enzyme 291
platelet-derived growth factor receptor (PDGF-R) 229
pleckstrin homology domain 266
pleiotropic redox mediator thioredoxin 213
polymerase chain reaction (PCR) 429
– amplification 446
– methods 445
– molecular biology techniques 443
– reaction 446
PLP-binding pockets 82
PLP-dependent enzyme, cysteine synthase 84
pneumococcal strains 384
polar surface area 104, 109
polar surface components 105
polyclonal/monoclonal antibodies 390
polysaccharide conjugate vaccine 391
polysaccharide intercellular adhesin 392
post-discovery synergies 10–11
post-genomic methods 386
post-translational modifications 267
potency-solubility-permeability triad 155
potential drug targets 227
– trypanosomatid protein kinases 227
PPDK inhibitor 80
PPi analogs 77
– active site 78
– carbonyldiphosphonate/bisphosphonates 77
PPi-dependent enzyme 77
– phosphofructokinase 77
praziquantal analogs 302, 305
– structure 304
predictive cell assays 434
pre-existing kinase-directed chemical libraries 241
presynaptic latrophilin-dependent pathway 349
primary lymphoid organ 164
procyclic trypanosomes 237
product development partnerships 34
promoter elements 379
promoter region, *see* Introns
prophylactic drugs 165
prophylactic/therapeutic vaccines 357
prophylactic treatment 166
protease inhibitors 184, 198
–approaches 180
protein biosynthesis 377, 382
protein-coupled receptors 19
Protein Data Bank (PDB), 3D structure database 50
protein expression 375
protein kinase drug discovery 250–251
protein kinase families 249
protein kinase inhibitors 240, 292
– multiple approaches 253
– opportunities/challenges 240
protein-ligand complexes 91, 330, 334
– interactions 333
proteins sequences 182, 288
– post-translational modifications 288
– targets 336
– 3D structures 336
protein-splicing elements 66
protozoan protein kinase targets 239, 251, 256
– Aurora kinases (AUK) 239, 240
– glycogen synthase kinase-3 (GSK-3) 239
protein kinases 218, 228, 230, 241, 264
– pharmacological inhibitors 230
protein phosphatase activity 228
proteome data 382
proteomic methods 291
proton motive force 360
protozoan blood parasite 159
– *Theileria parva* 159
protozoan parasite 6, 7, 184, 188, 197, 211, 241 277
– *Entamoeba histolytica* 184
– *Plasmodium falciparum* 277
– *Leishmania* 35
protozoan/helminth parasites 182
protozoan/mammalian kinases 241
protozoan/worm parasites 75
Pseudomonas putida 82
public-private partnerships (PPPs) 34, 117, 179, 302
– drug discovery 34, 117, 179, 302
pulmonary arteries 343
pulmonary infectious diseases 421
pulmonary lymph nodes 433
pulmonary surfactant 397
pulsed-field gel electrophoresis 443
purine-derived nitriles 190
putative components 196
putative cysteine proteases 182
putative papain-like protease, SERA8 179
putative peptidases 190
putative protein 182
pyruvate dehydrogenase 288, 290

pyruvate ferredoxin oxidoreductase (PFOR) 80, 81
– dehydrogenase 80
– H-bond network 81
– reaction 81
– recognition 80
– thiamine pyrophosphate cofactor 81
pyruvate-forming reaction 78
pyruvate oxidative decarboxylation 81
pyruvate phosphate dikinase 77

q

quantitative structure-activity relationship (QSAR) 47, 302
quinol-fumarate oxidoreductase (QFR) 361
quinone binding site 93
quinupristin/dalfopristin-resistant strains 399
quinone derivatives 93

r

radical derivatives 81
radiolabeled inhibitors 181
rational drug design 51
– development 160
– discovery programs 59
– SAM synthetase 51
RCSB Protein Data Bank 326
real-time PCR assays 185
receptor-ligand interactions 161
receptor-mediated endocytosis 189
recombinant DNA technology 169
recombinant enzymes 252
– oncosphere antigen 169
recombinant parasites 232
redox cyclers 212
Reformatsky aldol reaction 467
resistance-breaking anthelmintic compound 358
respiratory chain 104, 361
– anthelmintic drug target 359
– inhibitors 359
restriction enzymes 447
reticuloendothelial system (RES) 167
rhomboid intramembrane protease 179
rhomboid proteins 187
rhoptry protein 187
ribonucleotide reductase 217
– reduction 213, 217
ribosomal proteins 382
ring systems 303, 460
risk management 59
RNA-based hybridization 385
RNA dot blot analysis 182
RNA interference (RNAi) technique 12, 46, 65, 312
– systems 66
Roll Back Malaria (RBM) 278
root mean square deviation (RMSD) method 331, 53
– measures 331
– value 53
ruminant production systems 190
russujaponols, structures 459

s

S-adenosyl-L-methionine synthetase 51, 53, 54
– model structure 53, 54
– sequences 51
Saharan African countries 189
Salmonella infections 380
SAT isoforms 83
scanning electron microscopy (SEM) 216
schistosome parasites 311, 312
– drug discovery 315
– genome sequence 312
– redox cascade 312
Streptococcus pneumoniae 383
Schistosomiasis Research Unit 306
schizogony/gametocytogenesis 256
Schizosaccharomyces pombe cdc2 mutant 264
Scientific Advisory Committee (SAC) 37
scoring process 332
screening cascade 102, 106
screening library 109
semi-automated medium-throughput phenotypic screening 314
semisynthetic artemisinin derivatives 301
semisynthetic derivative 343
semisynthetic lipoglycopeptide 398
semisynthetic pristinamycin derivatives 399
sensory neurones 20
sequencing technologies 390
serine-aspartate (SD) repeat-containing proteins 392
– SdrD 392
– SdrE 392
– SdrG 392
serine *O*-acetyltransferase 82
serine proteases 183, 184, 198
– activities 179
– inhibitors 183, 198
serine proteinases 187
serine/threonine protein kinases 229, 234
sexually transmitted disease (STD) 182
– *Trichomonas vaginalis* 181
sheep gastrointestinal nematode 20

Shikimate biosynthetic pathway 89
– erythrose-4-phosphate 89
– phosphoenolpyruvate 89
shikimate kinase 418
sialic acid-binding protein 179
signaling pathways 253
signal peptides 196
signal transduction cascade 229, 347
single-dose therapies 313
single nucleotide polymorphism (SNP) 23
single strand conformation polymorphism (SSCP) 386
site-directed mutagenesis 84, 90
– experiments 86
sleeping disease, *see* HAT
SLO-1 channel subunit structural cartoon 349
SLO-1-dependent pathway 349, 352
SLO-1 pathway 20
– mutants 20
small molecule discovery center (SMDC) 316
SNIP-SNP mapping techniques 349
solid sclerotic granuloma 426
solvent-accessible surface areas 365
southern blot analysis 182
SPACE primers 446
spastic paralysis 20
species-specific targets 11
spindle-shaped flagellates 197
sporozoites 177
Staphylococcus aureus 391
state-of-the-art 301
– biochemical methodology 455
– validation system 66
stem cell factor receptor 229
structural/metabolic drug targets 18
structural/spectroscopic data 90
structure-activity relationship (SAR) 100, 254, 362
structure-based VS methods 327, 335
structure-related parameters 130
substrate binding sites 78
subtilisin-like serine protease 179
subunit vaccines 168
sugar acids 389
– galacturonate 389
– gluconate 389
sulfur-containing amino acids 81
– deamination 81
– dethiomethylation 81
– metabolism 76, 81, 84
superoxide radical 219
surface-associated proteins 388, 390
surrogate assays 40
Swern oxidation 462
synaptic proteins 238
syntenic relations gap closure 444
synthetic antibiotic, 453, 454
– fluoroquinolones 454
– prontosil 453
– quinolones 454
synthetic antiparasitic drugs 4
synthetic dyes 3
synthetic libraries 383
synthetic optimization 104
synthetic tetrahydroquinoline 308
– oxamniquine 308
synthetic trioxolanes 309, 310
– antischistosomal activity 310
systems biology/metagenomics 386, 387

t

T-helper cells 164
Theileria. annulata peptidases 190
– secretion 191–195
T4 DNA ligase 446
target-active compounds 62
target-associated toxicity 126
target-based antibacterial drug discovery 59, 99, 418
– approach 69
– assays 70, 100
– mode of action 69
– programs 60
target-based screening 99, 106, 107
– drug identification 99
– focused libraries 109
– lead toxicity 102
– library attrition 110
– library design 108
– programs 65
target-directed resistance mechanisms 17
target identification techniques 64–65
target-inhibitor complex 330
target parasites 62
target product profiles (TPPs) 38, 314
target selected treatment (TST) 26
target-specific toxicity 70
tautomeric/ionization states 328
TB 415, 432
– chemotherapy 432
– drug discovery 415, 421
TBDPS-ether 465
TCA cycle 284
TDR program 9
temperature-inducible GAL4 transcription activation 68
tetrahydropyrimidines pyrantel 340

thiol metabolism 86
thiol redox enzymes 312
thioredoxin glutathione reductase (TGR)-specific double-stranded RNA 312
thioredoxin-related protein 213
Theileria genome 190
– cell lines 197
Theileria peptidase 196
Theileria signalase 196
thiamine pyrophosphate (TPP) 80
– synthesis 289
thienopyrimidine analogues 362
thin-walled oocysts 188
tick drug 121
toll-like receptors (TLR) 164
Toxoplasma gondii 168, 186
– genome project 186
transcuticular diffusion 126
transdermal compounds 152
transgenic RNAi system 66
– induction 67
translational/rotational orientation 331
transmission electron microscopy (TEM) 216
transposon mutagenesis 65, 69
trans-sulfuration pathway 82
trehalose-6-phosphate synthase 327
Trichomonas vaginalis 181
trichostrongylid parasite 358
– *Haemonchus contortus* 358
– *Teladorsagia circumcincta* 358
tritium-labeled PF1022A 344
tRNA aminoacylation reaction 384
t-RNA synthetases 403
tropical diseases 120
– Chaga′s disease 120
– onchocerciasis 120
– schistosomiasis120
Trx-dependent pathways 312
TrxR/GR-dependent pathways 312
trypanocidal drugs 211
trypanosomal glutathione reductase 213
Trypanosoma cruzi 35, 180
– active site 87
– trypanothione reductase
trypanosomatid CDKs 235, 237
trypanosomatid family 211
trypanosomatid parasites 227, 235, 236
trypanosomatid protein kinases 227
trypanosomatid protozoa 86, 227, 228
– trypanothione metabolism 86
trypanosomatid species 228
– *Trypanosoma brucei* 177
trypanosome alternative oxidase (TAO) 87
trypanothione-dependent peroxidase system 214
trypanothione reductase (TryR) 86, 213, 312
trypanothione-related enzymes 215
– genetic manipulations 215
– inhibitors 219
trypanothione synthetase (TryS) 211, 213
trypanothione system 87, 212, 213, 214, 215, 216, 220
– inhibitors 220
– schematic presentation 214
TryR inhibitors 219, 221
TryS inhibitors 218, 219
TSH motif 262
tuberculosis 415, 432
– chemotherapy 432
– drug discovery 415, 421
– gaps 415
– issues 415
tuberculous necrotizing granuloma 429
tubulin-like GTPase, FtsZ 402
TXY-motifs 231, 232
tyrosine kinase-like kinases 229

u

UAS/GAL4 system 66, 67, 68
ubiquitin-conjugating enzyme 269
– PfUBC13 269
UCSF Sandler Center for Basic Research 315
– screen workflow 315
ultrasonic treatment 168
UNICEF 278
unicellular parasite 177
– plasmodium 177
United Nations Development Program 278
University of California SanFrancisco (UCSF) 314
α,β-unsaturated ester 462
urinary tract infection (UTI) 389

v

vaccination 166
vaccine adjuvants 170
vaccines/vaccination protocols 161
vancomycin-resistant enterococci (VRE) 403
variable surface glycoprotein (VSG) 238
vector-borne parasitic disease, HAT 189, 197
vector-transmitted diseases 165
ventral cord interneurones 20
vertebrate-specific neurotoxic protein 345
veterinary anthelmintic drug 120
veterinary anticoccidial drug 120
veterinary antiparasitic drug 118, 121, 127, 140, 147

– approaches 121
– discovery programs 7
– drug discovery 119, 135
– function-based approach 124–125
– historical strategies 118
– impact 7
– interactions 119
– mechanism-based approach 123–124
– physiology-based approach 125–126
veterinary-approved drugs 9
veterinary-driven development program 10
veterinary market 9
veterinary nematodes 358
veterinary oral ectoparasiticide 121
veterinary parasite 4, 5
veterinary pharmaceuticals 155
vinyl sulfone-based inhibitors 185, 186
viral vectors 169
virtual screening process 323, 326
– 2D property-based ligand profiles 324
– descriptor-based similarity measures 325
– pharmacophore approaches 325
– protein-ligand-based methods 325
virulence-associated genes 389
virulent strain 160
virus vaccines, development 160
visceral leishmaniasis 35, 36
– black sickness/kala-azar 35
voltage-activated potassium channels 349
voltage-gated calcium channel 19
voltage-gated potassium channel 20

w

Waals energy 333
Walter Reed Army Institute of Research (WRAIR) 305
web-linked based portal 41
whole-cell assays 8, 424
whole-genome shotgun sequencing 443
– projects 442
whole-organism targets 110
Wittig reaction 467
World Health Organization (WHO) 18, 212, 278, 227, 302, 313

x

XA-induced gamete formation 267
xestoquinone inhibitor 264
– PfPK5 264
X-ray crystallography 80
X-ray diffractrometry method 341
X-ray structures 326

y

Yersinia pestis 45

z

Ziehl-Neelsen acid-fast staining 425